Immunoinducible carbon dots-incorporated hydrogel as photothermal-derived antigen depot to trigger robust antitumor immune response

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Abstract

Photothermal therapy (PTT) causes immunogenic tumor cell death (ICD), while fails to elicit a strong anti-tumor immune response partially due to its inherent immunosuppressive microenvironment and low antigen presentation. To address these issues, we developed an immunoinducible carbon dots-incorporated hydrogels (iCD@Gel) through a dynamic covalent Schiff base reaction using mannose modified aluminum-doped carbon dots (M/A-CDs) as cross-linking agent. The M/A-CDs not only possessed superior photothermal conversion efficiency, but also served as nanocarrier to load CpG ODNs for inducing the maturation of dendritic cells (DCs) via mannose receptor-mediated targeting delivery. Upon intratumoral injection, the as-prepared iCD@Gel induced immunogenic tumor cell death and damage-related molecular patterns (DAMPs) release via photothermal ablation under 808 nm NIR irradiation. Subsequently, the iCD@Gel synergized with the DAMPs to significantly promote the maturation and antigen cross-presentation of DCs. Taken together, this work provides a promising strategy to develop nanoparticle-based therapeutic hydrogel for tumor immunotherapy.

Introduction

Photothermal therapy (PTT) synergized with immunotherapy has revolutionized the cancer treatment [1, 2]. In the process of tumor treatment, PTT not only leads to thermal ablation of tumor cells, but also triggers the anti-tumor immune response via the release of immunogenic cell death (ICD)-mediated damage-related molecular patterns (DAMPs) [3, 4]. These DAMPs facilitate the recruitment and activation of antigen presenting cells (APCs) to activate strongly T cell-mediated anti-tumor immune responses [5,6]. However, PTT-triggered ICD cannot inspire an intense immune response because of the intrinsic immunosuppressive microenvironment and low antigen presentation [7]. The introduction of immune adjuvants in cancer therapy is considered to an effective strategy to amplify the PTT-mediated anti-tumor immune response [8, 9].

It has demonstrated that immune adjuvants synergized with PTT could activate the APCs to trigger intense congenital and adaptive immune response [10]. Cytosine-phosphate-guanine oligodeoxynucleotide (CpG-ODN) is a TLR9 receptor agonist which located on the inner membrane of lysosome [11], and could activate DCs to induce humoral and cellular immunity [12-15]. It has been reported that CpG-ODN was the most effective immunostimulator at present, which was closely related to its structure [16-21]. Nevertheless, the electrostatic repulsion of free CPG-ODN resulted in low transmembrane transport efficiency and severely inhibited its immunomodulatory effect [22]. Therefore, it is urgent to develop suitable vector to delivery CpG-ODN in vivo for combined immunotherapy. Carbon dots (CDs) are emerging photoluminescence materials with many unique properties, including good biocompatibility, water solubility and easy functionalization [23, 24]. Therefore, CDs are widely used in various drug delivery systems, bioactive compounds and gene delivery systems [25]. Our previous work had proved that hyaluronate (HA) and polyethylenimine (PEI) functionalized carbon dots (HP-CDs) displayed great potential in nucleic acid loading capacity for gene delivery [26]. Meanwhile, CDs with

strong absorbance in the near infrared (NIR) region exhibited high photothermal conversion efficiency and might be used as ideal photosensitizer for PPT [27].

In addition, systemic using of immunotherapy drugs is difficult to avoid their non-specific biological distribution in normal tissues, while this nonspecific off-targeting of the immune system activates autoimmune-like damage to normal tissues. This phenomenon increases the risk of potential immune-related adverse events (irAEs) while limiting the effectiveness of drugs [28, 29]. Recently, mounting evidences demonstrated that in situ treatment of tumor could avoid this risk. Injectable hydrogel is an excellent drug-loading platform, which has good biocompatibility and none-immunogenicity. It can be in a flowing state before injection and rapidly form gel network in situ after injection, which shows great potential in biomedical applications [30, 31].

Inspired by the above statements, we aim to develop an in situ immunoinducible hydrogel based on the CDs for PTT and CpG-ODN delivery in vivo. Firstly, we prepared aluminum doped amino carbon quantum dots (A-CDs) using diethylenetriamine pentaacetic acid (DTPA) and the polyethyleneimine (PEI) as precursors. Then, the resultant A-CDs was modified by mannose for DCs targeting and loaded the CpG-ODNs to form the M/A-CDs@CpG. Finally, immunoinducible carbon dots-incorporated hydrogels (iCD@Gel) was fabricated through a dynamic covalent Schiff base reaction between amino groups of M/A-CDs@CpG and aldehyde groups of oxidized glucan [32]. We explored the photothermal performance and gene delivery capacity of M/A-CDs, as well as the impact of M/A-CDs@CpG treatment on the DCs maturation and antigen cross presentation in vitro. Using the M/A-CDs@CpG as cross-linker, we further studied that rheological properties of iCD@Gel. Afterward, we also demonstrated that the effect of on-site formation of iCD@Gel on the tumor growth and immune system via photothermal ablation and antitumor immune activation was investigated by 4T1 tumor bearing mice. Especially, we focused on the the DCs maturation and cytotoxic T lymphocyte (CD8⁺ T) profiles in vivo after treatment.

Methods

Materials

Polyethyleneimine (PEI), Glucan, Mannose, Triacetylamino sodium borohydride, Aluminum chloride, and hexahydrate ($\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$) were purchased from Aladdin Reagent Co., Ltd. (Shanghai). Diethylenetriaminepentaacetic acid (DTPA), Sodium periodate are purchased from McLin Reagent Co., Ltd. (Shanghai). Cell counting kit-8 (CCK-8) and 4,6-diamino-2-phenylindole (DAPI) were obtained from Thermo Fisher Scientific. Phosphate buffered saline (PBS) is purchased from Hyclone (Logan City, Utah, USA). Dulbecco minimum essential medium (DMEM), RPMI modified medium (RPMI-1640) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). Trypsin was from Sigma-Aldrich (USA). Deionized water was used in the experiment.

Synthesis of iCD@Gel

4 g of DTPA was dissolved in 30 ml deionized water and mixed with PEI solution (12.5 g dissolved in 200 ml). After microwave heating the supernatant was collected by centrifugation (3,000 r.p.m., 15 min). After dialysis CDs powder was obtained by freeze-drying. 4 g DTPA was dissolved in 30 ml deionized water and mixed with 30 ml $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ solution (0.1 g/mL) (solution 1). 200 ml PEI solution was mixed with the solution and reacted at room temperature. After microwave heating for the supernatant was collected by centrifugation (3,000 r.p.m., 15 min) after dialyzed and freeze-drying, A-CDs powder was obtained. A-CDs powder was mixed with acetamyl sodium borohydride and mannose at a mass ratio of 1 : 1 : 1 to slow stirring at room temperature. After dialyzed and vacuum freeze-drying, M/A-CDs precipitation was obtained. M/A-CDs@CpG was obtained by mixing CpG with M/A-CDs powder at a mass ratio of 1 : 100. To prepare the oxidized glucan, 3.24 g of glucan and 8.65 g of sodium periodate were dissolved in 70 mL of deionized water with stirring in the dark room for 20 min. Then, 1 mL of diethylene glycol was added to terminate the reaction. The obtained solution was dialyzed to obtain oxidized glucan powder by vacuum freeze-drying. To obtain the gel, 69.4 mg M/A-CDs@CpG was dissolved in 1 mL deionized water, and 80 mg oxidized dextran was dissolved in 1 mL deionized water, respectively. After mixing the two solutions, gel-like iCD@Gel could be obtained immediately.

Instruments and characterizations of iCD@Gel

The phase structures were analyzed by X-ray diffractometer (XRD) on Rigaku SmartLab 9 kW with a Cu K α radiation source ($\lambda = 0.15418$ nm), a slit of 0.5, and a scanning speed of 7/min. X-ray photoelectron spectroscopy (XPS) analysis was performed on Thermo Fisher Nexsa (USA). A560 dual beam uv-visible spectrophotometer (AOE Instruments, Shanghai) was used to identify the uv-visible absorption spectrum. The morphology of M/A-CDs@CpG was observed by transmission electron microscope (TEM, FEI Talos F200S, America).

Photothermal conversion efficiency

The photothermal conversion efficiency of M/A-CDs is calculated according to the method previously reported [33]. Under the irradiation of 2.0 W/cm² 808 nm near-infrared laser, the temperature change within 600 S was recorded, and then the temperature decline process within 600S was recorded after the laser was turned off. The photothermal conversion efficiency (η) was calculated according to the following formula (1)-(3).

$$\eta = \frac{hS(\Delta T_{\max, \text{mix}} - \Delta T_{\max, \text{H}_2\text{O}})}{I(1 - 10^{-A_{808}})} \quad (1)$$

$$hS = \frac{\sum_i m_i C_{p,i}}{t} \ln \theta \quad (2)$$

$$\theta = \frac{\Delta T}{\Delta T_{\max, \text{mix}}} \quad (3)$$

In the above formula, h is the heat transfer coefficient, S is the surface area of the container, $\Delta T_{\max, \text{mix}}$ is the temperature change of A-CDs at the maximum stable temperature. $\Delta T_{\max, \text{H}_2\text{O}}$ is the temperature change of water at its maximum stable temperature. I is the laser power, and A_{808} is the absorbance value of A-CDs in aqueous solution at 808 nm wavelength. θ is the dimensionless driving force temperature and is defined as the ratio of ΔT to $\Delta T_{\max, \text{mix}}$.

Gel retardation experiments

2.0 g of agarose was weighed and placed in a triangular flask. 100 mL 1 × TAE electrophoresis buffer was added and heated to complete dissolution in a microwave oven. When the solution temperature was cooled to about 50 °C, 1.1 μL of GelRed dye solution was added to mix well. A sufficient amount of TAE buffer was added into the electrophoresis tank to make the gel immersed in the liquid at a depth of about 1 cm. According to different mass ratio of CpG : M/A-CDs NPs (1 : 1, 1 : 2, 1 : 5, 1 : 10, 1 : 50, 1 : 100, 1 : 200, 1 : 500, 1 : 1000) this two solutions were mixed into complex and 7 μL solution, and then 1 μL loading solution was added. The samples were carefully added to the gel sample hole with a liquid shifter, and 8 μL of sample was added to each hole. Then the current was switched on, the voltage was 100 V. Stop electrophoresis about 30 minutes later and observe the fluorescent band under ultraviolet light.

Hemolysis test

After successful anesthesia, the mice's eyeballs were removed with forceps, and fresh blood from the eyeballs was collected into the heparin anticoagulant blood collection tube. The mouse red blood cells (MRBC) were collected after being cleaned for 3 times. The diluted MRBC (100 μL) was co-incubated with ACMC (4, 10, 15, 20, 25, 40, 80, 100 μg/mL) at room temperature. PBS was set as negative control and deionized water as positive control. After 2-6 hours, centrifugation at 1000 RPM for 1 minute, the absorbance of supernatant was measured by a dual-beam UV-vis spectrophotometer.

Biocompatibility of A-CDs

CCK-8 method was used to evaluate the biocompatibility of A-CDs. Different concentrations of CDs (0.05, 0.1, 0.2, 0.4, 0.8, 1, 5, 8, 10 μg/mL) were co-incubated with DC 2.4 cells and MEF cells (mouse embryonic fibroblast cell line) in 96-well plates (37 °C, 5 % CO₂), about 1×10⁴ cells per well. After 48 h incubation cells were incubated with 10 μL CCK-8 reagent and 90 μL DMEM medium. Then absorbance of the solution at 450 nm was measured by UV spectrophotometer (BioTek Instruments, Inc Vermont, USA). The experiment was repeated in three parallel groups. The relative cell viability was calculated as $(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{zero-sit}}) / (\text{Abs}_{\text{control}} - \text{Abs}_{\text{zero-sit}}) \times 100 \%$.

Phagocytosis experiment

The process and distribution of M/A-CDs@CpG NPs in cells were studied by confocal laser scanning. 4T1 cells were seeded at the bottom of the 24-well plate (10⁴ cells/well in one pass, 37 °C and 5 % carbon dioxide, 24 h). When the cell density reached 60 %, M/A-CDs NPs were added and cultured for 30 min.

After cleaning 3 times, the cells were fixed with 4% paraformaldehyde(room temperature, 15 min) and the nuclei were stained with DAPI working solution in dark room for 5 min. After strong cleaning, the small disk was taken out for production. Sealed with neutral resin and stored at 4 °C. Observe cells under laser confocal microscope.

In vitro immune analysis

DC 2.4 cells were seeded in 6-well plates (1×10^6 cells/well), cultured with different concentrations and different treatments of NPs at 37 °C and 5 % CO₂ to induce DC 2.4 cells maturation. After 48h, DC 2.4 cells were collected and stained with anti-CD86 PE, anti-CD80 APC, analyzed by flow cytometry. Another group of experiments, DC 2.4 cells and OVA + NPs co-culture, 48h after collecting DC 2.4 cells stained with H2kb-PE, flow cytometry analysis results.

Animals

Female Babl/c mice (18-22 g, 6-8 weeks old) were purchased from Changzhou Cavents Laboratory Animal Co., Ltd. The housing conditions for the animals with an ambient temperature of 24 °C and a relative humidity of 50 %.All animal operations in accordance with Jiangsu University Laboratory Animal Care and Use Guide.The 4 t1 tumor mice treated with drugs were divided into six groups, including PBS, CDs NPs, A-CDs NPs, M/A-CDs, M/A-CDs@CpG NPs and iCD@Gel. When the average size of the tumor reached about 100mm³, nano-drugs were injected in situ. (1mg/mouse in treatment group), hydrogel group was injected with double-channel syringe. The tumors were irradiated by 808 nm laser with a power density of 2 W/cm² for 5 minutes on the 1st, 3rd and 5th day, respectively. The tumor volume was monitored with vernier caliper every 2 days during treatment for 20 days. For immunological analysis, inguinal lymph nodes and spleens were isolated and made into single-cell suspensions after 7 days of treatment to 4T1 tumor mice. Inguinal lymph node cells suspensions and spleen cells suspensions were stained with APC anti-mouse CD3 (BioLegend), PE anti-mouse CD8a (BioLegend), APC anti-mouse CD11c, FITC anti-mouse CD80, PE anti-mouse CD86, respectively. The proportion of CD11c⁺CD80⁺CD86⁺ cells and CD3⁺CD8⁺ corresponding cells in DCs and effector memory T cells.

Histocompatibility

In order to study biocompatibility, the main organs (heart, spleen, liver, lung, kidney) of mice in different treatment groups were collected. Soak in 4 % paraformaldehyde buffer solution, then trim, dehydration, transparent treatment, paraffin embedding.Specimens were sliced continuously, thickness 5 μm, hematoxylin and eosin staining. Tissue sections were observed under an optical microscope.

Statistical analysis

Each experiment was repeated at least three times independently. All data are represented by mean standard deviation. Student t test was used for statistical analysis of the two groups, and single factor analysis of variance was used for difference between groups. P value was calculated by GraphPad Prism

8 software. The significant difference was expressed as $*P<0.05$, $**P<0.01$, $***P<0.001$ and $****P<0.0001$.

Results And Discussion

Preparation and characterization of the iCD@Gel

The procedure for the synthesis of iCD@Gel was illustrated in scheme 1. Briefly, the aluminum doped carbon dots (A-CDs) were synthesized by hydrothermal carbonization using PEI as passivator and metal chelating agent DTPA. Then, these A-CDs were surface modified by mannose as a targeting agent via chemical grafting to form M/A-CDs. Next, the M/A-CDs could be used as nanocarrier for loading immune adjuvant CpG ODNs by electrostatic adsorption to synthesize M/A-CDs@CpG. Finally, iCD@Gel was fabricated by the Schiff base reaction between amino groups on the M/A-CDs and aldehyde groups on the oxidized glucan.

The typical transmission electron microscopy (TEM) images showed that the resultant M/A-CDs were spherical with an average diameter of approximate 4.3 nm (**Fig. 1a and b**). This size allowed them to stay in the blood circulation for a long time and passively accumulate in tumors through enhanced permeability and retention (EPR) effects [34]. It could be found from the X-ray diffraction pattern in **Fig. 1c** that a wide diffraction peak appeared at 2 θ of 25, which proved that M/A-CDs has amorphous phase structure. The chemical composition of M/A-CDs was characterized by FTIR and XPS. The FTIR spectra of M/A-CDs showed (**Fig. 1d**) that a wide absorption peak appeared near 3300 cm^{-1} , which was ascribed to -OH stretching vibration. The asymmetric stretching vibration peak of $-\text{CH}_2$ appeared near 2926 cm^{-1} , and the peak at 1613 cm^{-1} belonged to the scissors bending vibration of $-\text{NH}_2$. Two small and sharp peaks at 1460 cm^{-1} and 1465 cm^{-1} belonged to the antisymmetric bending vibration peak of $-\text{CH}_3$ groups. The peak near 1283 cm^{-1} corresponded to the bending vibration signal frequency of $-\text{CH}$, and the peak at 1092 cm^{-1} corresponded to the vibration contraction of $-\text{C}-\text{O}$. These results showed that there are many hydrophilic groups on the surface of M/A-CDs, which endowed M/A-CDs with superior stability and dispersion in water. In addition, the introduction of nitrogen element could significantly improve the luminescence intensity, that was meaningful to the multifunctional applications in bioimaging. The full XPS spectra in **Fig. 1e** showed three typical peaks, C1s (284.55 eV), N1s (398.62 eV) and O1s (531.64 eV) with the contents of 77.91 % (C), 10.8 % (N) and 10.79 % (O), respectively. The atomic ratios of N/C and O/C were 44.6 % and 83.22 %, (**Fig. 1 f-i**), indicating that A-CDs were highly doped with the element of N and O. At the same time, the existence of aluminum was verified by two small peaks at 84.98 eV-65.18 eV, and the content was 0.5 %. This result proved that aluminum has been doped into the CDs in an appropriate proportion. To verify the grafting of mannose, the nuclear magnetic hydrogen spectrum of M/A-CDs was detected. It could be seen (**Fig. 2a**) that the same shape peak appeared at the same position in the nuclear magnetic hydrogen spectrum of M/A-CDs sample and pure mannose, which proved that the mannose was successfully grafted to the surface of A-CDs.

The gene loading capacity of M/A-CDs was evaluated using gel retardation assay. We envisioned that CpG ODNs with negatively charge could move to the negative electrode in agarose gel electrophoresis, but this migration could be reversed when the charge was neutralized by M/A-CDs (**Fig. 2b**). The results (**Fig. 2c**) showed that the CpG ODNs bands could still be observed clearly in the positive electrode when the mass ratio of CpG ODNs : M/A-CDs was less than 1:5. However, the bands became lighter when the mass ratio decreased to 1:10, indicating that only a small amount of CpG ODNs bound with M/A-CDs and failed to migrate. When the mass ratio decreased to 1: 50, CpG ODNs bands were completely blocked to the opposite direction of migration. The tendency became strong with the decreasing mass ratio. In **Fig.2d**, the results showed that zeta potential of CpG ODNs was -29.2 mV and zeta potential of M/A-CDs was 27.9 mV. Whereas zeta potential of CpG ODNs mixed with M/A-CDs was 11.7 mV, which further confirmed M/A-CDs bound with CpG ODNs. These results proved that the M/A-CDs possessed the ability to carry the CpG ODNs for targeted delivery.

Hydrogel itself as ideal delivery mode has profound impacts on the efficiency of immune activation [35]. Therefore, we attempted to develop an in situ hydrogel using the M/A-CDs as cross-linker to further enhance their immune response. As we expected, the amino groups on the surface of M/A-CDs and the aldehyde group in side chain of dextran underwent aldoamine condensation via the schiff base reaction, leading to the rapid formation of hydrogel (iCD@Gel) in the **Fig. 2e**. The morphology of hydrogel was observed by SEM (**Fig. 2f**), it was found that iCD@Gel had a typical sponge scaffold appearance. This dense network structure plays an important role in the performance of hydrogels and biomedical engineering applications. The internal interaction of hydrogels is one of the key factors affecting cell growth and drug release [36]. Afterward, the rheological properties of iCD@Gel was generally characterized by Young's modulus. The amplitude scan (**Fig. 2g**) shown the dependence of storage modulus G' and loss modulus G'' of iCD@Gel on the applied strain (γ %). The result indicated that the storage modulus G' was significantly higher than the loss modulus G'' in a large range, showing that iCD@Gel possessed a gel organization. In addition, iCD@Gel had elasticity up to 12010 % strain at a fixed angular frequency of 1 Hz before the gels collapsed ($G'' > G'$). which proved that the iCD@Gel had extraordinary mechanical properties. The extraordinary mechanical strength may be ascribed to the contribution of the imine bond network within the iCD@Gel [37-39].

Optical characteristics of M/A-CDs

Given the intrinsic property of CDs, the optical characteristics of M/A-CDs was analyzed by UV-Vis spectrum and PL spectrum (**Fig. 3a**). It can be seen from **Fig. 3c** that the PL emission of M/A-CDs reached a peak at 462.9 nm under the 346 nm excitation wavelength. When excitation wavelength increased from 300 nm to 500 nm, the emission had a wide range from 450 nm to 650 nm, the red shift was obvious due to the photon reabsorption (**Fig. 3b**). These results proved that the M/A-CDs we prepared were different from traditional fluorescent substances because they had excitation-light-dependent emission characteristics. In the UV absorption spectra of M/A-CDs (**Fig. 3d**), there was strong optical absorption in the ultraviolet region due to the π - π^* transition of the C = C bond on their surface. The maximum absorption peak at about 320 nm and extending to the visible region. Meanwhile, M/A-

CDs showed bright blue fluorescence under UV light irradiation, which verified their favorable fluorescence performance.

Photothermal property of M/A-CDs

Mounting evidence demonstrated that CDs exhibited favorable photothermal behavior due to the nonspecific absorption in near infrared region, but their photothermal conversion efficiency (PCE) were inefficient. Therefore, we tended to improve the photothermal performance of CDs through heteratomic doping strategy. The results shown that the PCE of M/A-CDs was higher than traditional CDs, indicating that aluminum doping could significantly promote the PCE of CDs in **Fig. 4a**. Based on the calculation formula from previously publications [40], the photothermal conversion efficiency of M/A-CDs was 37.7 %, which was much higher than those of bare CDs without aluminum ions (13.3 %) (**Fig. 4b-e**). These findings shown that the incorporation of metal elements could enhance the photothermal effect of CDs significantly. This phenomenon may be attributed to the doping of metal elements producing a new electron transition path inside the CDs, thereby enhancing the radiation heat efficiency [41]. Subsequently, the photothermal performance of M/A-CDs in aqueous solution (1.5 mg/mL) was studied under different power densities. As shown in **Fig. 4f**, the temperature of M/A-CDs solution increased sharply with the laser power increased. It was reported that hyperthermal could induce intense apoptosis of tumor cells when the temperature reached 55 °C. Under the same laser power (2 W/cm²), different concentrations of M/A-CDs irradiation solution also showed excellent photothermal effect (**Fig. 4g**). During this period, the heating imaging of M/A-CDs was recorded by the infrared camera, which was consistent with temperature curve (**Fig. 4h**). These results showed that the M/A-CDs could convert the NIR laser energy into thermal energy effectively in a concentration-dependent manner. As shown in **Fig. 4i**, the temperature curve of the traditional photosensitizer indocyanine green (ICG) gradually decreased after the five laser on/off cycles. In contrast, the temperature curves of M/A-CDs remained during same time, which indicating that M/A-CDs has excellent photothermal stability. Besides, the introduction of aluminum ions may be used as traditional immune adjuvant and facilitate anti-tumor immune activation.

Biocompatibility, intracellular uptake and photothermal ablation of M/A-CDs

The biocompatibility characterization of biomaterials is very essential before biomedical applications. **As shown in Fig. 5a and b**, the presence of two absorption peaks at 560 nm after water treatment indicated hemolysis. On the contrary, different concentrations of M/A-CDs had little effect on the blood cells. The cytotoxicity of M/A-CDs in vitro was detected by standard CCK-8 method. The DC 2.4 and MEF cells were co-cultured with M/A-CDs at different concentrations for 48 h, respectively. The result shown that both of cells viability were maintained above 95 % after treatment, indicating the favorable cytocompatibility of M/A-CDs (**Fig. 5c**). Then, M/A-CDs was evaluated as a photothermal agent for ablation of cancer cells in vitro under laser irradiation. 4T1 cells were exposed to 1.5 W/cm² power density of 808 nm laser after incubation with different concentrations of M/A-CDs for 4 h. The results showed that cells viability obviously decreased with the increase of M/A-CDs concentration under NIR laser irradiation (**Fig. 5d**). Taken together, these results clearly verified that M/A-CDs possessed excellent biocompatibility and

photothermal performance of promoting tumor cells ablation in vitro. The phagocytosis and subcellular localization of M/A-CDs in 4T1 cells were observed using different organelle probes by confocal fluorescence microscopy. As shown in **Fig. 5e**, the green fluorescence derived from M/A-CDs could be observed in the cytoplasm after co-cultured for 4h. After being labelled by different organelle probes, the strong orange-yellow fluorescence could be observed in lysosomes and mitochondria. This preference distribution may be ascribed to positive charge nature of M/A-CDs.

Immune activation function of M/A-CDs@CpG in vitro

DCs with powerful antigen-presenting ability play key role in anti-tumor immunity [42]. Hence, we studied the effect of M/A-CDs@CpG on the maturation and antigen-presenting ability of DCs according to the principle in **Fig. 6a**. Initially, the CD80 and CD86 expression of DCs in the A-CDs group (11.4 %) was 2.5 times higher than control group (4.5 %) in the **Fig. 6b** and **c**. Notably, the CD80 and CD86 expression of DCs in the M/A-CDs@CpG group was up to 17.8 %, indicating that the M/A-CDs@CpG possessed a strong ability to induce DCs maturation. Meanwhile, the CD80 and CD86 expression increased with the increase of M/A-CDs@CpG concentration from 6.3 % to 19.8 % (**Fig. 6d and e**), further confirming the above results. It is well known that antigen presentation by classical MHC class I molecules to CD8⁺ T cells is a central aspect of the adaptive immune response. Hence, we choose SIINFEKL peptides derived from OVA as model molecules to exploit the antigen cross presentation ability of DCs. As shown in **Fig. 6f and g**, the H-2Kb-SIINFEKL peptide expression (32.4 %) in M/A-CDs@CpG group could be detected by flow cytometry, which was higher than that in control group (17.9 %), CDs group (24.5 %), A-CDs group (25.2 %), M/A-CDs group (28.4 %). It could be seen that M/A-CDs@CpG exhibited the strong ability to promote antigen cross-presentation performance of DCs.

The anti-tumor effect of iCD @Gel in vivo

Encouraged by the excellent PPT and immune activation performance in vitro, we further explored the inhibition of iCD@Gel on tumor through tumor-bearing mice (**Fig. 7a**). Upon 808 nm laser irradiation, the tumor temperature in all treatment groups increased rapidly after 5 mins except PBS group (**Fig. 7b and c**). It was worth noting that A-CDs exhibited higher photothermal performance than CDs, which was ascribed to introduction of aluminum. Among them, iCD@Gel not only raised the temperature of tumors from 26 °C to 56 °C within 5 min, but also stayed inside the tumor for up to 5 days (**Fig. 7d**). These results proved that the iCD@Gel possessed superior photothermal performance and long term retention in vivo, which was conducive to extend therapeutic window and persistent immunostimulation.

Subsequently, we investigated the therapeutic effect of iCD@Gel using the 4T1 tumor-bearing mice. The results shown that CDs-mediated PTT in the CDs, A-CDs, M/A-CDs, M/A-CDs@CpG, iCD@Gel groups significantly suppressed the growth of tumors, compared with that in PBS group. Impressively, the tumor in the iCD@Gel group was almost eradicated and did not relapse within 20 days (**Fig. 7e and f**). The survival time of mice in iCD@Gel group was extended by more than 60 days (**Fig. 7g**). In addition, weight fluctuations in all groups throughout the treatment process could be negligible, which confirmed that

these biomaterials have no significant adverse effects on mice (**Fig. 7h**). At last, we assessed the long-term potential biocompatibility by the histopathological section when the experiments was terminated. It was apparently that no pathological changes or inflammatory could be observed in major organs (**Fig. 7i**). These results indicated that iCD@Gel with high photothermal performance has potential to serve as a novel therapeutic method in precision therapy of tumor in situ.

The anti-tumor immune activation of iCD@Gel in vivo

The strong anti-tumor immune activation is essential to prevent tumor recurrence and metastasis. Therefore, the anti-tumor immune activation of the iCD@Gel in vivo were evaluated through analysis of the DCs maturation and CD8⁺ cytotoxic T lymphocytes (CTLs) differetiation. After distinct treatments, the lymph nodes and spleen tissues were collected for flow cytometry analysis. Consistent with the results in vitro, the iCD@Gel triggered the higher expression level of CD80⁺/CD86⁺ DCs than the other treatments (**Fig. 8a**). Specifically, the ratio of CD80⁺CD86⁺ DCs in the lymph nodes in PBS, CDs, A-CDs, M/A-CDs, M/A-CDs@CpG, iCD@Gel treatment were 1.6 %, 2.8 %, 4.4 %, 10.8 %, 15 % (**Fig. 8b**). The ratio of CD80⁺CD86⁺ DCs in the spleen in different treatments gradually increased from 0.7 % to 5 % (**Fig. 8e**), which was similar to the trend in lymph nodes. These phenomenons may be ascribed to the PTT-triggered DAMPs release and CpG ODNs stimulation. Generally, the iCD@Gel exhibited the outstanding ability to induce DCs maturation. Afterward, the CD8⁺ cytotoxic T cells in the lymph nodes was measured after treatments. The results shown that the ratio of CD8⁺ T cells in the lymph nodes in PBS, CDs, A-CDs, M/A-CDs, M/A-CDs@CpG, iCD@Gel treatment was 24.7 %, 31.7 %, 33.5 %, 39 %, 42.2 % and 48.8 % (**Fig. 8f and g**). Compared with the treatment groups that free of hydrogels, iCD@Gel induced more higher frequency of CD8⁺ T cells, indicating positive effect of hydrogels to the immune activation. The identical trend of percentage of CD8⁺ T cells in spleens could be observed from 14 % to 41.5 % (**Fig. 8h and i**). All of these results demonstrated that iCD@Gel significantly increased the frequency of CD8⁺ T cells in the spleen and the lymph nodes, which was conducive to anti-tumor immune response.

Conclusion

In this work, we developed a novel in situ iCD@Gel for combined photothermal immunotherapy of cancer. The resultant iCD@Gel was composed of versatile M/A-CDs and aldehyde glucan. It was found that the introduction of aluminum into M/A-CDs greatly improved their photothermal conversion efficiency (37.7%), which was higher than traditional CDs (13.3%). In addition, M/A-CDs with mannose modification could be served as nanocarrier to load CpG ODNs for inducing the maturation of DCs via manose receptor-mediated targeting delivery. Upon intratumoral injection, the iCD@Gel was in situ formed instantly via the M/A-CDs cross-linked aldehyde glucan based on Schiff base reaction. As we expected, the iCD@Gel induced immunogenic tumor cell death and DAMPs release via photothermal ablation under 808 nm NIR irradiation. More importantly, the iCD@Gel synergized with the DAMPs to significantly promote the maturation and antigen cross-presentation of DCs. These results indicated that the iCD@Gel

has the potential to integrate photothermal ablation and anti-tumor immune activation for individualized precision therapy of tumor.

Declarations

Ethics approval and consent to participate

All experimental procedures were approved by the Animal Care and Use Committee of Medical College of Jiangsu University (No. UJS-IACUC-AP-20190314002).

Consent for publication

Each co-author has read the manuscript and approved its submission, and the work will be submitted to your journal.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SL, one of the main authors, was responsible for the preparation and characterization of nanomaterials. JL was in charge of cell culture. XX, HW and YW assisted in cell experiment and flow cytometry. M and QL assisted in the animal experiments. All authors read and approved the final manuscript.

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Figures

Figure 1

(a) Typical TEM images of M/A-CDs. Scale bar: 20 nm. (b) Particle size distribution, (c) XRD spectra, (d) FTIR spectroscopy, (e) XPS spectra of M/A-CDs. Peak-fitting spectra for O1s (f), C1s (g), N1s (h), Al 2ps (i) of M/A-CDs.

Figure 2

(a) NMR images of M/A-CDs. **(b)** Schematic diagram of CpG ODNs loaded on M/A-CDs. **(c)** Gel retardation assay of M/A-CDs and CpG ODNs with various mass ratios. **(d)** Zeta potential of M/A-CDs@CpG (1: CpG, 2: M/A-CDs, 3: M/A-CDs:CpG=100:1). **(e)** Comparative photos of iCD@Gel before and after gelation. **(f)** Typical SEM images of iCD@Gel, **(g)** The strain amplitude sweep test at a fixed angular frequency (1 Hz) at 37 °C.

Figure 3

Optical properties of M/A-CDs. **(a)** Photoluminescence spectra of M/A-CDs at different excitation wavelengths from 300 nm to 500 nm (PL). **(b)** Normalized PL spectra of M/A-CDs. **(c)** Maximum emission spectra of M/A-CDs at maximum excitation wavelength. **(d)** UV-visible absorption spectra of M/A-CDs.

Figure 4

Photothermal performance of M/A-CDs. **(a)** The temperature curves of the M/A-CDs and the CDs. **(b)** The temperature curve of CDs solution treated with an NIR laser (808 nm, 2 W/cm²) for 600 s and left to cool down and **(c)** linear time data versus -lnθ obtained from the cooling stage. **(d)** The temperature curve of M/A-CDs solution treated with an NIR laser (808 nm, 2 W/cm²) for 600 s and left to cool down and **(e)** linear time data versus -lnθ obtained from the cooling stage. **(f)** The temperature curves of M/A-CDs (1.5 mg/mL) solution under NIR irradiation of 808 nm with different power densities (0-2.5 W/cm²). **(g)** The temperature curves of M/A-CDs solution with different concentrations (0-2 mg/mL) under NIR irradiation of 2 W/m² power density. **(h)** Thermal images of M/A-CDs solution under different laser power density. **(i)** The temperature curves of M/A-CDs solution after five laser on/off cycles.

Figure 5

(a) Blood compatibility of M/A-CDs at different concentrations and **(b)** following amplified version (530-560 nm). P, PBS. W, water. **(c)** Cytocompatibility of M/A-CDs at different concentrations. **(d)** Effect of M/A-CDs on 4T1 cells under 808 nm laser irradiation at power of 2 W/m². **(e)** Fluorescence images of 4T1 cells co-cultured with M/A-CDs after different organelle probe labeling.

Figure 6

The regulation of M/A-CDs@CpG on DC cells maturation. **(a)** Schematic illustration of the experimental design. Representative flow cytometry plots **(b)** and the statistic data **(c)** of the CD 80⁺86⁺ on the surface of DC2.4 cells after different treatments (1, Blank. 2, CDs. 3, A-CDs. 4, M/A-CDs. 5, M/A-CDs@CpG). Representative flow cytometry plots **(d)** and **(e)** statistic data of CD80⁺86⁺ on the surface of DC2.4 cells after co-cultured with different concentrations of M/A-CDs@CpG (1, Blank. 2, 0.2 μg/ml. 3, 0.5 μg/ml. 4, 1.0 μg/ml. 5, 5.0 μg/ml) . **(f)** Flow pattern and **(g)** Quantitative analysis of H2kb antigen expression on the surface of DC2.4 cells after different treatments (1: Blank, 2: CDs, 3: A-CDs, 4: M/A-CDs, 5: M/A-CDs@CpG). Data are presented as the mean ± s.d. Statistical significances were calculated by one-way analysis of variance with Tukey's post hoc test and two-tailed Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 7

In vivo photothermal effect of iCD@Gel and its inhibitory effect on tumor growth. **(a)** Schematic illustration and experimental design. **(b)** Thermal images of mice after in situ injection of iCD@Gel under 808 nm laser irradiation with a power density of 1.5 W/cm² at different time points (1, PBS. 2, CDs. 3, A-CDs. 4, M/A-CDs. 5, M/A-CDs@CpG. 6, iCD@Gel). **(c)** Temperature curves of different treatment groups at different time points under 808 nm near-infrared laser irradiation (1 mg/kg). **(d)** Temperature changes of different treatment groups after 5 days under 808 nm near-infrared laser irradiation. **(e)** Tumor volume curves of each mouse within 20 days of treatment. **(f)** Average tumor volume curves each mouse within 20 days of treatment. (n = 5). **(g)** Body-weight change of mice in different groups. **(h)** Survival curves of mice in the different groups. **(i)** H&E-stained images of the major organs in the different treatment groups. Scale bar: 200 μm. ***P<0.001, ****P<0.0001.

Figure 8

The immunoregulatory effect of iCD@Gel in vivo. **(a)** Experimental design. **(b)** The representative flow cytometry and **(c)** quantitative analysis of CD80⁺CD86⁺ cells from the lymph node. **(d)** The representative flow cytometry and **(e)** quantitative analysis of CD80⁺CD86⁺ cells from the spleens. **(f)** The representative flow cytometry and **(g)** quantitative analysis of CD3⁺CD8⁺ T cells from the lymph node. **(h)** The representative flow cytometry and **(i)** quantitative analysis of CD3⁺CD8⁺ T cells from spleens. (Groups: 1, Blank. 2, CDs. 3, A-CDs. 4, M/A-CDs. 5, M/A-CDs@CpG. 6, iCD@Gel.)

Supplementary Files

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- [Scheme1.png](#)