

Construction of PAMAM-based Nanocomplex Conjugated with Pt(IV)-complex and Lauric Acid Exerting Both Anti-tumor and Antibacterial Effects

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Electronic Supplementary Information

Abstract Oxaliplatin is the common chemotherapy drug for the treatment of colorectal cancer (CRC), however, the harmful *Fusobacterium nucleatum* (*Fn*) that existed in the CRC tumor tissues leads to extreme drug resistance to oxaliplatin, which reduces the anticancer efficiency. Here we fabricated a new dendrimer-based nanocomplex with anti-tumor and antibacterial dual-functions through conjugating Pt(IV)-complex and lauric acid (LA) to the dendritic carrier PAMAM (G=3). The synthesized PAMAM-Pt-LA nanoparticle was further coated with hyaluronic acid (HA) on its surface through electrostatically interaction in order to enhance biocompatibility and tumor targeting. The biocompatibility, anti-tumor activity and antibacterial activity of PAMAM-Pt-LA@HA nanocomplex were evaluated *in vitro*. The results showed that the PAMAM-Pt-LA@HA nanocomplex exhibited a good biocompatibility, high anti-colorectal cancer activity and high anti-*Fn* activity. This dual-functional nanocomplex provides a new method to develop anticancer nanomedicines that will be more therapeutically efficacious and less toxic than oxaliplatin for the treatment of CRC.

Keywords Colorectal cancer; *Fusobacterium nucleatum*; Anti-tumor and antibacterial; Drug resistance; PAMAM-based nanoparticles

Citation: Xin, Y. T.; Wang, L. Y.; Chang, H. H.; Ma, F. H.; Sun, M. L.; Chen, L.; Gao, H. Construction of PAMAM-based nanocomplex conjugated with Pt(IV)-complex and lauric acid exerting both anti-tumor and antibacterial effects. *Chinese J. Polym. Sci.* 2023, 41, 887–896.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers and the second leading cause of cancer-related death all over the world.^[1] In recent years, the mortality and morbidity of colon cancer are still increasing.^[2] The 5-year survival rate of CRC patients is only 8%–10% at the advanced stage.^[3] Chemotherapy is one of the most important methods in the treatment of CRC clinically.^[4] To date, many chemotherapeutic agents have been developed to treat CRC, such as 5-fluorouracil (5-FU), capecitabine, palitaxel (taxol), doxorubicin, camptothecin and platinum compounds.^[5–7] Among them, the third-generation platinum(II) anticancer drug oxaliplatin that was approved in 2002 was most commonly used in the treatment of CRC.^[8]

Oxaliplatin inhibits the synthesis of DNA, RNA and protein

of cancer cells, and triggers cancer cell apoptosis through oxidative stress.^[9] However, due to its toxicity and the occurrence of drug resistance, the therapeutic index of oxaliplatin is narrow, which restricts its clinical application. To date, many approaches have been developed to improve the efficacy and reduce the toxicity of oxaliplatin. One of the most promising approach is the prodrug strategy, that using platinum drugs with an oxidation state +IV, which has a lower ligand-exchange rate, as compared with the platinum(II) counterparts.^[8] However, the traditional low-molecular-weight drugs commonly have rather low tumor selectivity and accumulation. To overcome this problem, a variety of nano-drug delivery systems have been developed, such as dendrimers, liposomes, nano-carriers and polymer micelles.^[10,11] Compared with other systems, dendrimers are widely used due to their unique structure and narrow size, such as poly(L-lysine), poly(propylene imine) (PPI) and poly-amidoamine (PAMAM).^[12] Among them, PAMAM dendrimers have shown broad prospects as drug delivery systems due to their monodisperse, three-dimensional hyper-branched and stable structure, and easily modified surface active groups.^[12,13] Moreover, PAMAM can facilitate accumulation of

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Received September 17, 2022; Accepted October 20, 2022; Published online December 12, 2022

drugs to the tumor site through the "enhanced permeability and retention" (EPR) effect due to its suitable size (1–100 nm), which can reduce the side effects of the drug.^[14] Therefore, conjugating Pt(IV)-complex to PAMAM may be an ideal delivery system to treat CRC.

It has been reported that *Fusobacterium nucleatum* (*Fn*), one of the gram-negative anaerobic bacteria that was enriched in both stools and tumor tissues of CRC patients, can promote chemoresistance to CRC by modulating autophagy.^[15,16] Moreover, *Fn* also promotes the proliferation and metastasis of CRC. Therefore, elimination of the harmful *Fn* is significant to treat the CRC. It has been reported that lauric acid (LA) and its derivatives exhibits good antibacterial activity against both gram-negative and gram-positive pathogens through incorporating into the bacterial cell membrane.^[17] Thus, we hypothesize that by introducing LA to PAMAM, the resulted system could also exhibit high anti-*Fn* activity. In addition, the active tumor targeting activity of delivery systems should also be engineered in order to reduce the toxicity to normal tissues and enhance the anti-tumor efficiency. Hyaluronic acid (HA), one of a glycosaminoglycan with negatively charge on its surface, can recognize the over-expressed CD44 receptor located on the surface of colorectal cancer cells.^[18] It has been reported that by introducing HA to the surface of the nano-drug delivery system could prolong its blood circulation times.^[19] In our previous study, we also successfully constructed a HA/PEGylated amphiphilic delivery system for selective intracellular doxorubicin release.^[20] Therefore, HA was employed by coating negatively charged HA to the surface of positively charged PAMAM nanoparticles, which is expected to provide the tumor targeting property to the nanoparticles but also improve the biocompatibility of the delivery system by neutralized the positively charged

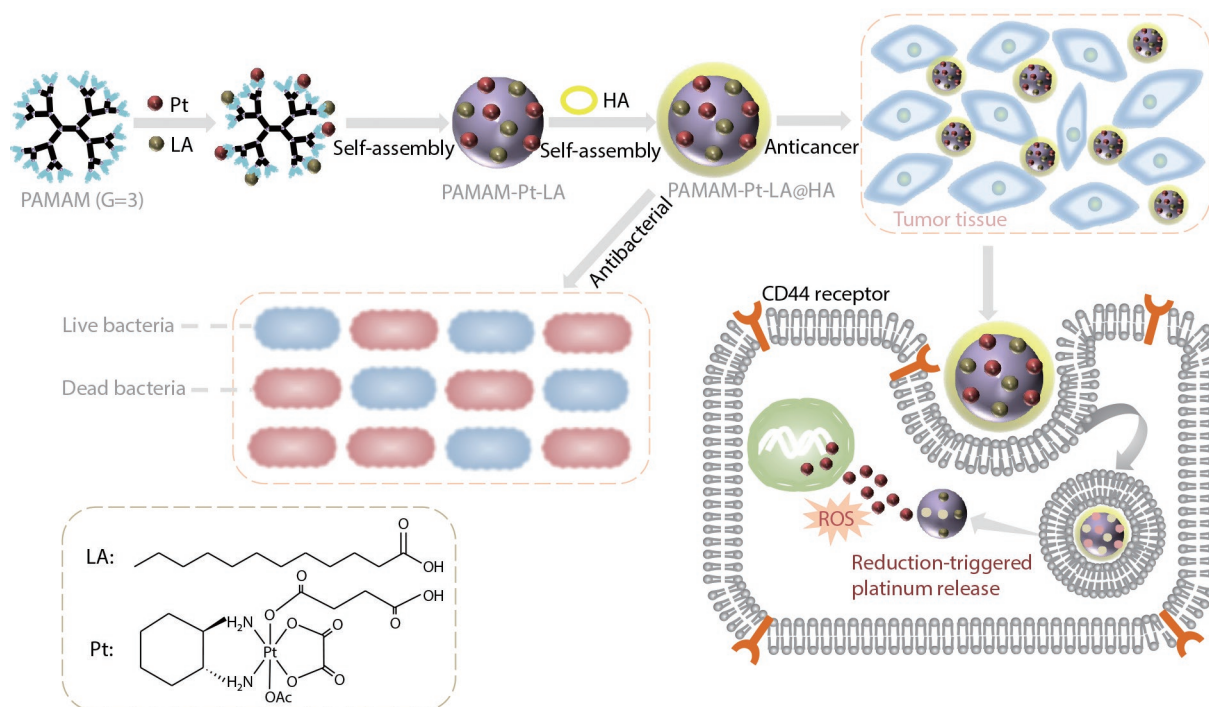
PAMAM nanoparticles.

Here, we engineered a novel nanocomplex with anti-tumor and antibacterial dual functions through conjugating both Pt(IV)-complex and LA to PAMAM (Scheme 1). We used the third generation PAMAM as it is relatively less toxic to cells compared with higher generation ones.^[21] The Pt(IV)-complex with carboxyl group and LA was first conjugated to PAMAM through reacting with amine on the PAMAM. Next, the resulted polymers were self-assembly in water to form PAMAM-Pt-LA nanoparticles as LA is hydrophobic in water. Finally, the negatively charged HA was coated on the surface of the PAMAM-Pt-LA nanoparticle through electrostatic interaction. The resulted PAMAM-Pt-LA@HA nanocomplex was characterized by scanning electron microscopy (SEM), dynamic light scattering (DLS), and zeta potential. The anti-cancer effect of PAMAM-Pt-LA@HA on HCT116 cells and the biocompatibility of PAMAM-Pt-LA@HA to L929 cells were evaluated *in vitro*. The antibacterial effect of PAMAM-Pt-LA@HA on *Fn* was also evaluated under anaerobic conditions *in vitro*.

EXPERIMENTAL

General Considerations

All moisture/oxygen sensitive reactions were performed using standard glove box or Schlenk techniques under N₂. Toluene, *n*-hexane, THF and Et₂O were dried by refluxing over sodium/benzophenone. CH₂Cl₂ was dried over CaH₂, distilled under N₂ and stored over activated molecular sieves (4A). CDCl₃ dried over CaH₂ and C₆D₆ dried over Na/K were vacuum transferred prior to use. AlMe₃ purchased from Aldrich and *rac*-LA purchased from TCI were used as received. ε-CL was purchased from Aladdin and dried over CaH₂. ¹H- and ¹³C-NMR spectra were recorded on a Bruker DMX-500 (500 MHz for ¹H,



Scheme 1 Schematic illustration of the fabrication of PAMAM-Pt-LA@HA nanocomplex for both anti-tumor and antibacterial *in vitro*.

125 MHz for ^{13}C) instrument. The gel permeation chromatography (GPC) measurements were performed on the instrument of Agilent 1260 Infinity using THF as eluent (40 °C and 1 mL·min $^{-1}$).

Synthesis of Monocarboxyplatinum

Monocarboxyplatinum was synthesized according to the previous report as shown in Scheme S1 in the electronic supplementary information (ESI).^[8] Briefly, oxaliplatin (400 mg, 1 mmol) was dissolved in acetic acid (8 mL), followed by addition of hydrogen peroxide (0.08 mL). The mixed solution was stirred at room temperature, and hydrogen peroxide (0.08 mL, 0.24 mL) was added continuously in an interval of 30 min. Then, the mixture was constantly stirred at room temperature and protected from light for 12 h. The reaction product was purified according to the following steps. Vacuum rotary evaporation to remove excess acetic acid and concentrate the crude product under reduced pressure, silica gel column chromatography ((EtOAc/MeOH, 3/1) to purify the crude product, and vacuum drying overnight to obtain compound 1 as the white solid powder product (250 mg, 50%). Then, compound 1 (200 mg, 0.43 mmol) and succinic anhydride (101 mg, 1.01 mmol) were dissolved in anhydrous DMF (6.67 mL), and then reacted at 50 °C overnight in dark. The solvent was removed by vacuum rotary evaporation under reduced pressure. Next, the product was dispersed in acetone (8 mL) and sonicated for 5 min. The supernatant was removed by centrifugation, and the precipitate was continuously dissolved in acetone (repeated for 3 times). The precipitate was washed with ice diethyl ether, stirred at room temperature for 20 min, and centrifuged at 12000 r·min $^{-1}$ for 10 min, followed by vacuum drying overnight to obtain compound 2 as the white solid powder product (120 mg, 65%). The chemical structure was verified by ^1H -NMR.

Synthesis of PAMAM-Pt-LA

The carboxyl-to-amine crosslinking was synthesized using EDC and *N*-hydroxysuccinimide (NHS) (Scheme S2 in ESI). Briefly, EDC (53.4 mg, 0.28 mmol) and NHS (32.2 mg, 0.28 mmol) were dissolved in 5 mL of anhydrous methanol under stirring, followed by adding of compound 2 (27 mg, 0.024 mmol) and Lauric acid (5 mg, 0.024 mmol). After stirring for 1 h, PAMAM (G=3) in methanol (1.5 μmol , 60 μL) was added. Then the mixture was reacted at room temperature for 12 h. After reaction, the mixture was added dropwise to ice anhydrous ether, stirred for 20 min, and then centrifuged at 12000 r·min $^{-1}$ for 10 min. Then collected the white precipitate and dissolved in deionized water. The solution was further dialyzed against methanol solution for 1 day and distilled water for another 2 days (MW cut-off: 3500 Da). The final product as light-yellow powder was obtained by freeze-drying with a yield of 60%, and characterized by ^1H -NMR.

Fabrication of PAMAM-Pt-LA@HA Nanocomplex

First, PAMAM-Pt-LA and HA were dissolved in ultra-pure water and the concentration was 1 mg/mL, respectively. Then, the resulted HA and PAMAM-Pt-LA solutions were mixed at different volume ratios (1/1, 1/2, 1/3, etc.) at room temperature for 30 min to get PAMAM-Pt-LA@HA nanocomplex.

Field-Emission Scanning Electron Microscopy (SEM)

SEM was measured at an accelerate voltage of 300 kV using a JEOL JSM-6700F microscope (Japan). The samples (0.5 mg/mL)

were ultrasonic dispersed in PBS and dried in air. Au coating of nanoparticles for imaging was carried out by sputtering for 90 s.

Particle Size and Zeta Potential Measurements of Nanoparticles

The size and zeta potentials of nanoparticles were estimated in three independent experiments by Zetasizer Nano ZS90 instrument (Malvern Instruments, Southborough, MA) at 25 °C. Briefly, PAMAM-Pt-LA (1 mg) or PAMAM-Pt-LA@HA (1 mg) was dissolved in deionized water (1 mL) to measure the particle size. PAMAM-Pt-LA (0.1 mg) or PAMAM-Pt-LA@HA (0.1 mg) was dissolved in deionized water (1 mL) to measure the zeta potentials (ζ -potential).

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Experiment

PAMAM-Pt-LA (1 mg) was dissolved in 60% HNO_3 (2 mL), and digested for 12 h at 80 °C. After digestion, the blank solution, standard solution, and samples were prepared. Typically, 60% HNO_3 (1 mL) was added into the deionized water (49 mL) to obtain the 2% HNO_3 solution. The blank solution was consisted of 2% HNO_3 solution (10 mL). Pt standard solution (6.8 μL) was added to 2% HNO_3 solution (20 mL) to obtain 0.34 $\mu\text{g}\cdot\text{mL}^{-1}$ standard solution. Standard solution (0.34 $\mu\text{g}\cdot\text{mL}^{-1}$, 5 mL) was added to 2% HNO_3 solution (5 mL) to get 0.17 $\mu\text{g}\cdot\text{mL}^{-1}$ standard solution. Standard solution (0.17 $\mu\text{g}\cdot\text{mL}^{-1}$, 2.5 mL) was added to 2% HNO_3 solution (7.5 mL) to obtain a 0.085 $\mu\text{g}\cdot\text{mL}^{-1}$ standard solution. The digested sample solution (6.8 μL) was added into deionized water (980 μL) and 2% HNO_3 solution (9 mL) to obtain the sample solution. All solutions were filtered by 0.22 μm water film. Then samples were measured using ICP-MS (Thermo Fisher Scientific Co., Ltd., Germany).

Hydrolysis Kinetics of PAMAM-Pt-LA

In order to determine the degree of hydrolysis of PAMAM-Pt-LA, PAMAM-Pt-LA sample solution (5 $\text{mg}\cdot\text{mL}^{-1}$) was prepared and divided into five groups (2 mL in each group), and then was injected into a preswelled dialysis tube with a molecular weight of 3.5 kDa. Then, the samples were soaked in 5 mL of PBS buffer solution (pH 5.0, 10 mmol/L) and shaken in an incubator at 37 °C. Enzyme was added into the PAMAM-Pt-LA solution as a stimulus-induced release factor. Samples were taken out at certain time intervals, freeze-dry and the degree of hydrolysis was analyzed by ^1H -NMR.

Drug Release

To examine the OxPt release profiles of PAMAM-Pt-LA@HA *in vitro*, 2.0 mL of PAMAM-Pt-LA@HA (1 $\text{mg}\cdot\text{mL}^{-1}$) solution was placed in a preswelled dialysis tube (3.5 kDa MW). Meanwhile, 2 mL of PAMAM-Pt-LA@HA (1 $\text{mg}\cdot\text{mL}^{-1}$) was placed in three different conditions: (1) pH 7.4; (2) pH 5.0 + HAase; (3) pH 5.0 + HAase + GSH; Collect samples at specific time intervals and add fresh media. Finally, the Pt content of each sample was calculated by ICP-MS. Similarly, for detecting the LA release spectrum of PAMAM-Pt-LA@HA, PAMAM-Pt-LA@HA (1 $\text{mg}\cdot\text{mL}^{-1}$) was placed in two different conditions: (1) pH 7.4; (2) pH 5.0 + HAase. the LA content of each sample was calculated by HPLC, and each sample was measured for 3 times in parallel.

Cytotoxicity Assay

The cytotoxicity of PAMAM, PtCOOH, PAMAM-Pt-LA and PAMAM-Pt-LA@HA against Mouse fibroblasts cells (L929 cells),

Normal human colon mucosal epithelial cell line (NCM460 cells), rat myocardial cells (H9C2 cells) or human colon cancer cells (HCT116 cells) was measured by methyl thiazolyl tetrazolium (MTT) assay. Briefly, the cells were first collected from culture flasks. Then, the cells were seeded into 96-well plates (5×10^3 per well) and incubated at 37 °C in a 5% CO₂ atmosphere for 24 h. Then the culture medium was replaced with 100 µL of fresh medium containing 10 µL of drug solution, followed by incubation for another 24 h. After co-incubation, the culture medium was cleared away and washed with 100 µL of PBS for once in each well, followed by addition of 90 µL of medium with 10 µL of MTT storage solution (5 mg·mL⁻¹ in PBS buffer). The MTT medium was cautiously removed after incubation for 4 h and washed with 100 µL of PBS for once in each well. Then, 100 µL of DMSO was added into each well to dissolve the formed crystals. Finally, the absorbance rate was measured at 570 nm using a microplate reader (Epoch, BioTek, Genecompany Limited). The percentage of cell viability was calculated by equation $(\text{Abs}_{\text{test cell}})/(\text{Abs}_{\text{control cell}}) \times 100\%$.

Fn was resuscitated and diluted (OD₆₀₀=0.1), then the diluted bacterial solution was mixed with pure 1640 Medium to obtain a cell culture solution containing *Fn*. The HCT116 cells were seeded in a 96-well plate at a density of 5×10^3 cells per well for culture. After culturing for a certain time, the original medium was removed and replaced with *Fn*-containing medium. After 3 h, the liquid in the plate was replaced with fresh medium containing 10% FBS. After an additional 3 h of culturing, HCT116/*Fn* were treated with different samples. Finally, cell viability was detected by MTT assay.

HCT116 cells were seeded in 24-well plates at a density of 1×10^6 cells per well and incubated for 24 h in 1640 Medium supplemented with 10% FBS. Cells were then washed for 3 times with PBS and infected with *Fn*. PAMAM-Pt-LA@HA particles were suspended in complete medium at the indicated concentrations and added to the well plate. After culturing for 24 h, the bacteria were collected by washing three times with PBS. Viable colony forming units (CFU) of bacteria were determined by spreading different dilutions onto blood plates and incubating at 37 °C for 24 h.

Reactive Oxygen Species Measurement

Dichlorofluorescein diacetate (DCF-DA) was used to detect the ability of drugs to induce ROS generation against cells. Briefly, DCF-DA (4.87 mg) was dissolved in NaOH (1 mL, 0.01 N) and stirred for 0.5 h at room temperature. The resulted solution was then neutralized with PBS (9 mL, 0.01 mol/L, pH=7.4) and stored in ice until further use. The cells were seeded on a 35 mm glass bottom dish with a density of 7×10^4 cells in 1 mL of RPMI 1640 medium supplemented with 10% FBS and cultured for 24 h, followed by replacement of the primary culture media with 1 mL of serum-free medium containing free Pt or Pt-loaded nanoparticles (at a final Pt concentration of 40 µmol/L). After incubating for 6 h, cells were washed for once by PBS (1X, pH=7.4), followed by adding 1 mL of serum-free medium and 10 µL of the above DCF-DA solution, and incubated for 30 min. Subsequently, the stained cells were washed with PBS (1X, pH=7.4) for three times and applied for fluorescence imaging under a light source of 10 MW·cm⁻². The fluorescence image of the samples was captured by confocal laser scanning microscopy (CLSM) (Zeiss LSM510, Oberkochen, Germany). The signal of DCFH-DA was excited at 488 nm, and the emission

light was collected in the range of 500–530 nm. No background fluorescence of cells was detected under the setting condition.

Evaluation of *In vitro* Antibacterial Effect

Fn was seeded to the BHI culture medium (5 mL) and shook at 37 °C overnight. The bacterial concentration was quantified by measuring optical density at $\lambda=600$ nm on a UV-Vis instrument. Then the cultured bacteria were diluted to glass tubes with 1 mL of PBS (pH=7.4) to adjust the OD₆₀₀ to 0.01. After that, PAMAM, PAMAM-Pt-LA and PAMAM-Pt-LA@HA were added into the prepared bacteria suspension, and the tubes were kept shaking (170 r·min⁻¹) at 37 °C for 4 h. After that, the bacterial suspensions were taken out and diluted, followed by transferring 100 µL of the bacterial dilution to the solid nutrient agar plate and incubated for 12 h at 37 °C, and the colonies were counted. PBS solution was used as a control. The bacterial viability rates were determined by the ratio of colony counts to the blank groups.

Statistics Analysis

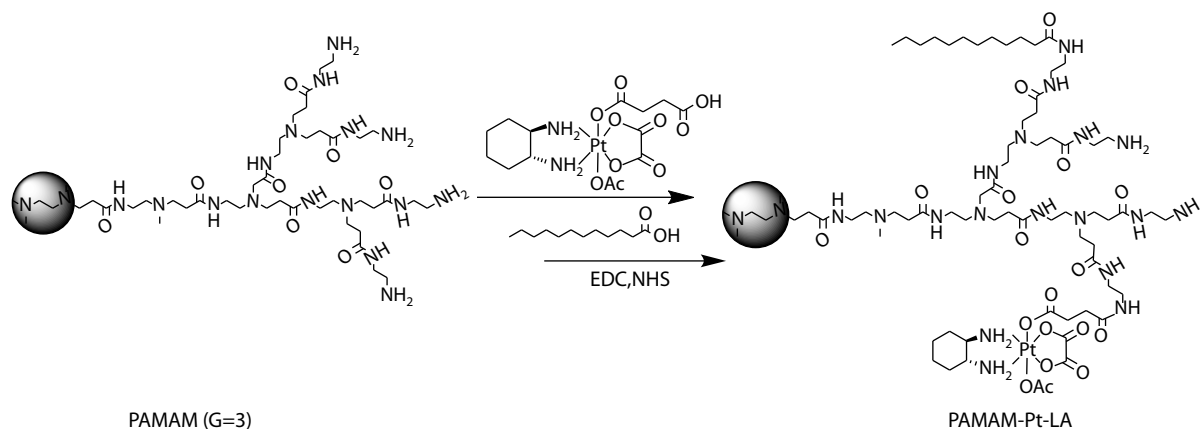
Significant differences in cell viability between any two groups were evaluated using a Student's t-test.

RESULTS AND DISCUSSION

Synthesis and Characterization

The monocarboxyplatinum was synthesized and characterized by ¹H-NMR as shown in Scheme S1 and Fig. S1 (in ESI). The polymer PAMAM-Pt-LA was synthesized as shown in Scheme 2 and characterized by ¹H-NMR (Fig. 1). According to the ¹H-NMR spectrum of PAMAM-Pt-LA, it can be calculated that six molecules of LA were conjugated to one molecule of PAMAM polymer. The content of platinum loaded on the PAMAM-Pt-LA polymer could be measured by ICP-MS. The measurement results showed that the platinum content was 198 µg/1mg PAMAM-Pt-LA polymer. The successful fabrication of PAMAM-Pt-LA nanoparticles and PAMAM-Pt-LA@HA nanocomplex were confirmed by DLS, SEM and zeta potential. As shown in Fig. 2(a), the PAMAM-Pt-LA nanoparticle has a narrow size distribution with the hydrodynamic diameter of 140±5 nm. After coating with HA (the volume of PAMAM-Pt-LA and HA was 1:1), the hydrodynamic diameter of PAMAM-Pt-LA@HA nanocomplex increases to 245±12 nm (Fig. 2b), which indicated that the HA was successfully coated to the surface of PAMAM-Pt-LA nanoparticles. The morphology and size of PAMAM-Pt-LA and PAMAM-Pt-LA@HA were observed by SEM. As shown in Figs. 2(c) and 2(d), the PAMAM-Pt-LA and PAMAM-Pt-LA@HA nanoparticles are uniform spheres with the average diameters of 130±8 and 240±7 nm, respectively. The particle size and PDI of PAMAM-Pt-LA and PAMAM-Pt-LA@HA solutions were also measured at different time points. As shown in Fig. 2(e) and 2(f), the solutions are stable with negligible changes of particle size and PDI, indicating that the formulations were very stable during storage.

The zeta potential measurement also confirmed the formation of PAMAM-Pt-LA@HA nanocomplex, that after coating with HA, the zeta potential changes from +14.6 mV to -26 mV as the negatively charged HA was coated on the surface of positively charged PAMAM (Fig. 3). After adding hyaluronidase, the zeta potential changed to +8.1 mV. This result indicated that in the presence of hyaluronidase, the coated hya-



Scheme 2 Synthesis route to PAMAM-Pt-LA.

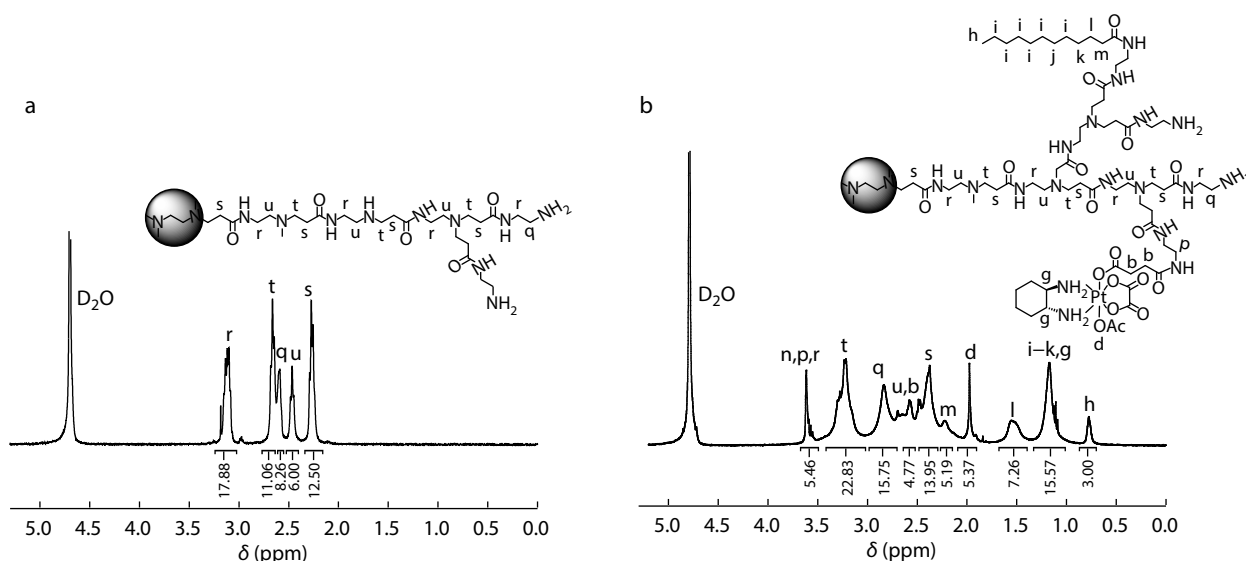


Fig. 1 ^1H -NMR spectra of (a) PAMAM (G=3.0) and (b) PAMAM-Pt-LA in D_2O .

luronic acid of the PAMAM-Pt-LA@HA nanocomposite would be decomposed to expose PAMAM-Pt-LA. It is beneficial for PAMAM-Pt-LA@HA to better exert antibacterial effects.

Generally, low molecular weight chemotherapeutic agents (<1000 Da) exhibit shorter blood half-life and lower levels of tumor accumulation compare with nanodrug systems.^[22] This is because that nanomedicines can reduce kidney excretion and accumulate in tumors *via* the EPR effect. Although the understanding about EPR effects is disputed in recent years,^[23–25] many reports showed that nanoparticles With a hydrodynamic diameter of 100–400 nm were considered optimal for tumor targeting.^[26,27] Moreover, negatively charged nanoparticles generally have prolonged circulation time.^[28,29] After being conjugated to PAMAM and coated with HA, the low molecular weight platinum drug was converted to nanomedicine with a hydrodynamic diameter about 240 nm and negatively charged surface, which should be more advanced for CRC therapy than oxaliplatin.

In vitro Biocompatibility

To evaluate the *in vitro* biocompatibility of PAMAM-Pt-LA@HA to normal tissues cells, the MTT assay was used to evaluate the

cell viability of L929, H9C2 and NCM460 cells after being treated with different drug formulations. As shown in Fig. 4, the carrier PAMAM has slightly obvious cytotoxic to all the 3 types of normal tissues cells, indicating that PAMAM-Pt-LA@HA was biologically safe for normal cells and a good choice to serve as the backbone to fabricate the nanomedicine systems. Both the combined monocarboxyl oxaliplatin and PAMAM-Pt-LA nanodrug show an apparent toxic effect on the 3 types of normal tissues cells at the concentration of 100 $\mu\text{mol/L}$, for which the corresponding cell survival rates were around 50%. It should be noticed that the cytotoxicity of PAMAM-Pt-LA to normal cells was lower than that of monocarboxyl oxaliplatin, and this maybe because that the platinum drugs could not be fully released from PAMAM-Pt-LA under the internal environment of normal cells. In comparison, after being coated with HA, the resulted PAMAM-Pt-LA@HA nanocomplex shows negligible cytotoxic to the 3 types of normal tissues cells that the cell survival rates were higher than 90% at the concentration of 100 $\mu\text{mol/L}$. These results demonstrated that coating of HA to the surface of PAMAM-Pt-LA highly improved the biocompatibility, which was crucial for the clinical application of

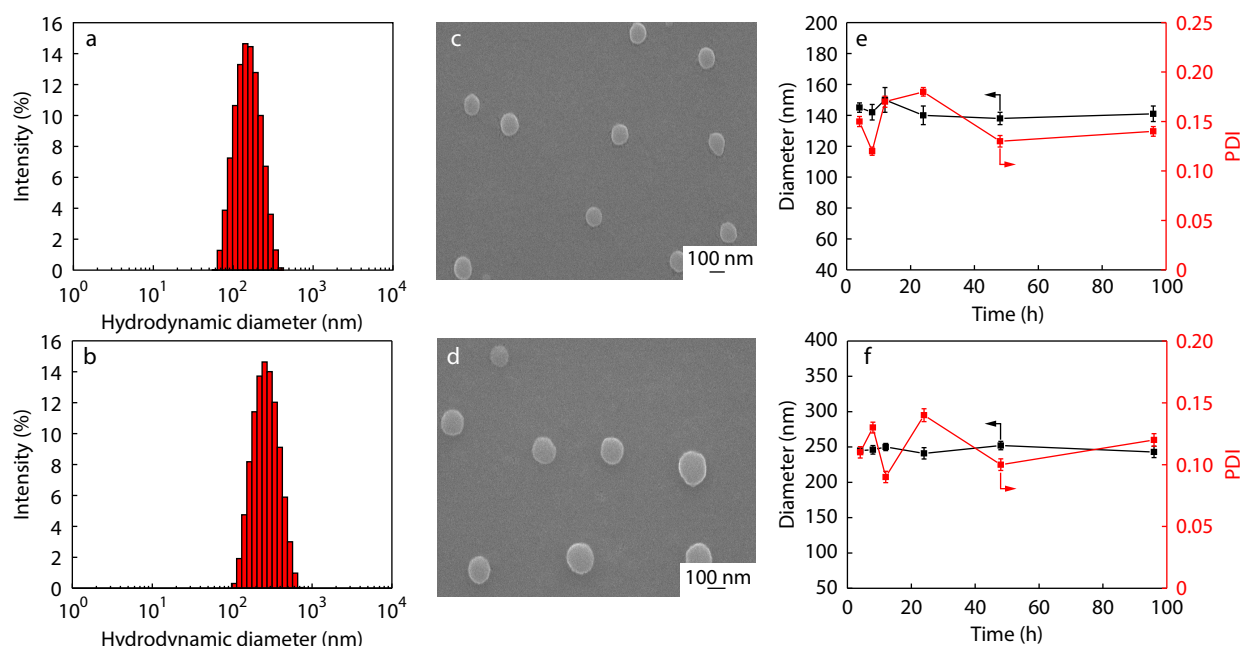


Fig. 2 Characterization of PAMAM-Pt-LA and PAMAM-Pt-LA@HA. (a, b) The hydrodynamic diameters and distributions of (a) PAMAM-Pt-LA and (b) PAMAM-Pt-LA@HA determined by DLS; (c, d) SEM images of (c) PAMAM-Pt-LA and (d) PAMAM-Pt-LA@HA; (e, f) Hydrodynamic size and PDI of (e) PAMAM-Pt-LA ($n=3$) and (f) PAMAM-Pt-LA@HA after preparation were measured in the following 96 h determined by DLS ($n=3$).

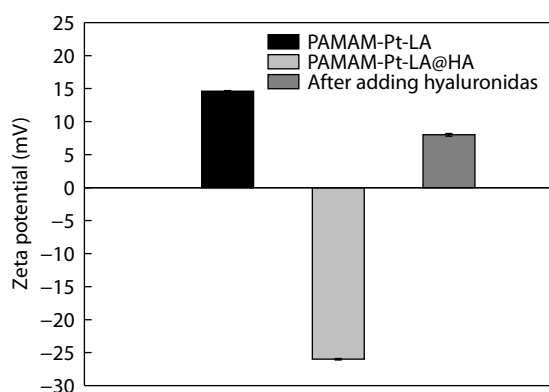


Fig. 3 Zeta-potentials of PAMAM-Pt-LA, PAMAM-Pt-LA@HA and the PAMAM-Pt-LA@HA after adding hyaluronidase ($n=3$).

nanomedicines.^[20]

In vitro Anticancer Activity

To evaluate the *in vitro* anticancer ability of PAMAM-Pt-LA@HA, the cytotoxicity of different drug formulations against HCT116 cells was analyzed by MTT method. As shown in Fig. 5, the free PAMAM has slightly obvious cytotoxic to HCT116 cells. The PtCOOH and PAMAM-Pt-LA show an apparent cytotoxic to HCT116 cells, which was similar with that to normal cells. In contrast, the PAMAM-Pt-LA@HA show the highest cytotoxic to HCT116 cells, while has the lowest cytotoxic to normal cells. This property of PAMAM-Pt-LA@HA is significant for tumor targeting delivery and to reduce the side effects to normal tissues. The reason may be attributed to the targeting effect of HA to the CD44 receptor which was highly expressed on the surface of colorectal cancer cell but not on normal cells.^[18,20] In addition, Pt(IV) could be reduced to Pt(II) under the reduction

environment in cancer cells, which led to an increased anti-tumor activity of platinum.^[8] On the basis of these findings, PAMAM-Pt-LA@HA showed high promising to treat CRC *in vivo*.

ROS Generation Induced by PAMAM-Pt-LA@HA

The generation of ROS is important for platinum-based drugs to induce cell apoptosis.^[30] ROS may participate in the process of cell death when cells produce excessive ROS.^[31] According to previous reports, platinum-based compounds generated ROS through oxidative stress and induce cytotoxicity.^[32] After cells being treated with anticancer drugs, the generated ROS could be intuitively detected using the DCFH-DA indicator, which would show green fluorescence when observed by confocal laser microscope. As shown in the Fig. 6, both H9C2 and HCT116 cells treated with PBS show invisible green fluorescence, indicating that the normal oxidative metabolism of normal cells or cancer cells produces nearly no ROS.^[33] In comparison, the H9C2 and HCT116 cells treated with PtCOOH show strong green fluorescence, indicating that the free platinum drugs could induce both normal and cancer cell apoptosis through generation of excessive ROS, which also indicated that the PAMAM-Pt-LA had no selectivity to cancer cells. Meanwhile, the fluorescence intensity of cells treated with PAMAM-Pt-LA groups is lower than that of PtCOOH groups, which indicated that less amount of PAMAM-Pt-LA entered into cells than the free platinum drugs. However, after coated with HA, the resulted PAMAM-Pt-LA@HA nanocomplex induces excessive ROS to HCT116 cells that the cells showed very strong green fluorescence. Moreover, the normal H9C2 cells treated with PAMAM-Pt-LA@HA show very weak green fluorescence, indicating that the PAMAM-Pt-LA@HA induced negligible generation of ROS. These results demonstrated that the HA could not only enhance uptake of cancer cells through targeting

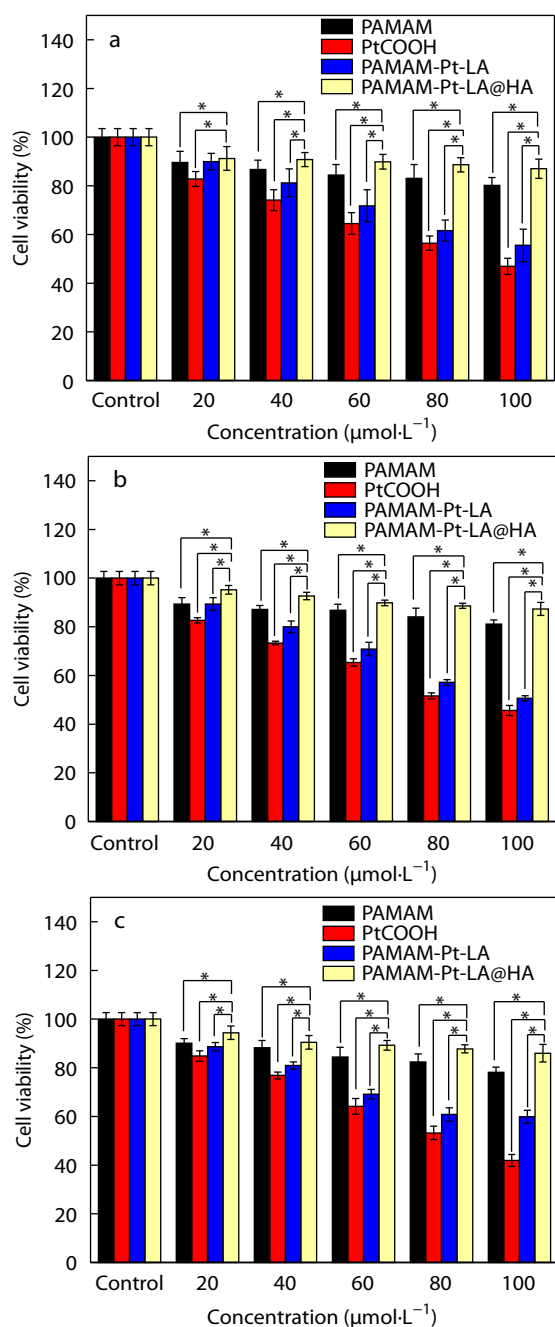


Fig. 4 *In vitro* cell viability of (a) L929 fibroblasts, (b) H9C2 cells and (c) NCM460 cells against PAMAM, PtCOOH, PAMAM-Pt-LA and PAMAM-Pt-LA@HA at different platinum concentrations after 24 h of incubation. The concentration of PAMAM in PAMAM group was the same as the concentration of PAMAM in PAMAM-Pt-LA and PAMAM-Pt-LA@HA groups. Mean $\pm$ SD, $n=3$. * $P<0.05$.

the CD44 receptor, but also reduce uptake of normal cells due to its negatively charged property.

Antibacterial Activity of PAMAM-Pt-LA@HA Against *Fn*

It has been reported that LA had antibacterial activity against *Fn*.^[34] Thus, we hypothesized that the PAMAM-Pt-LA@HA nanocomplex also had antibacterial activity when LA was

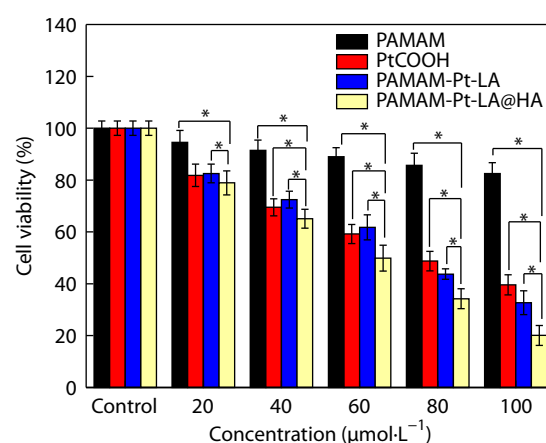


Fig. 5 *In vitro* cell viability of HCT116 cells against PAMAM, PtCOOH, PAMAM-Pt-LA and PAMAM-Pt-LA@HA at different platinum concentrations after 24 h of incubation. The concentration of PAMAM in PAMAM group was the same as the concentration of PAMAM in PAMAM-Pt-LA and PAMAM-Pt-LA@HA groups. Mean $\pm$ SD, $n=3$. * $P<0.05$.

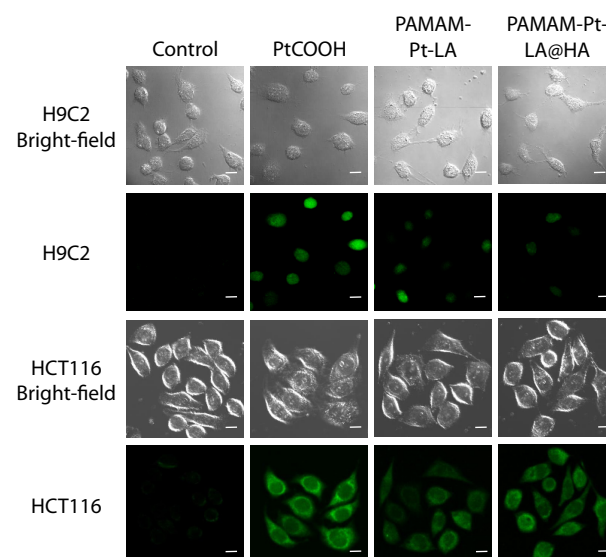


Fig. 6 The fluorescence microscope images of H9C2 or HCT116 cells treated with PBS (Control), PtCOOH, PAMAM-Pt-LA or PAMAM-Pt-LA@HA for 12 h at the platinum concentration of 40 $\mu\text{mol}\cdot\text{L}^{-1}$. After incubation with 10 $\mu\text{mol}\cdot\text{L}^{-1}$ DCFH-DA, cells were washed and examined by fluorescence microscope. Scale bar: 20 μm .

covalently linked to PAMAM, as the bacteria could secrete hyaluronidase,^[35,36] which can decompose the coated HA and induce the release of antibacterial LA. To evaluate the antibacterial activity of PAMAM-Pt-LA@HA against *Fn*, we used the flat coating method (Fig. 7 and Figs. S2–S5 in ESI). As summarized in Fig. 8, both the backbone carrier PAMAM and free platinum drug (PtCOOH group) have low antibacterial activity against *Fn*. In comparison, the antibacterial activity of free LA, PAMAM-Pt-LA and PAMAM-Pt-LA@HA against *Fn* increased significantly when increasing the concentration of LA. The survival rate of *Fn* is nearly 0% when the LA concentration is 100 $\mu\text{g}\cdot\text{mL}^{-1}$. Moreover, the antibacterial activity of free LA was slightly higher than that of PAMAM-Pt-LA@HA with different LA conjugating ratios at the same concentration of LA (Fig. S6 in

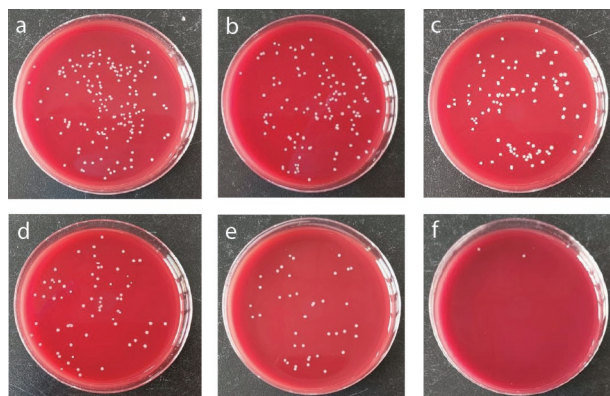


Fig. 7 Colony forming units (CFU) for *Fn* treated with PAMAM-Pt-LA@HA at different LA concentrations: (a) control group without any treatment, (b) 20 $\mu\text{g}\cdot\text{mL}^{-1}$, (c) 40 $\mu\text{g}\cdot\text{mL}^{-1}$, (d) 60 $\mu\text{g}\cdot\text{mL}^{-1}$, (e) 80 $\mu\text{g}\cdot\text{mL}^{-1}$ and (f) 100 $\mu\text{g}\cdot\text{mL}^{-1}$.

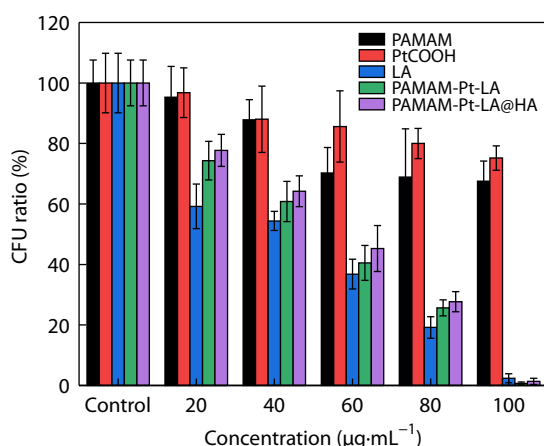


Fig. 8 The antibacterial activity of PAMAM, PtCOOH, LA, PAMAM-Pt-LA and PAMAM-Pt-LA@HA at different concentrations of LA (20–100 $\mu\text{g}\cdot\text{mL}^{-1}$). The concentrations of PAMAM of PAMAM group and PtCOOH of PtCOOH group were the same as the concentrations of PAMAM-Pt-LA and PAMAM-Pt-LA@HA groups ($n=3$).

ESI), indicating the insufficient release of LA from the nanoparticles which was confirmed by the result of the hydrolyzation kinetics of enzyme against PAMAM-Pt-LA (Fig. S7 in ESI).

PAMAM-Pt-LA@HA Reverses Drug Resistance Caused by *Fn*

As *Fn* accounts for chemotherapy resistance in CRC patients, we further investigated the anticancer effects of PAMAM-Pt-LA@HA on *Fn* co-cultured HCT116 cells. As shown in Fig. 9, the cell viability of HCT116 cells is significantly higher in the presence of *Fn* after treatment with PtCOOH than that of without *Fn* co-culturing, indicating that *Fn* induced drug resistance to PtCOOH. However, no significant difference can be found in the cell viability of HCT116 cells regardless in the presence of *Fn* or not after treatment with PAMAM-Pt-LA@HA. This result mainly depends on the release of Pt and LA in tumor microenvironment. As shown in Fig. S8 (in ESI), Pt and LA reach the maximum release in the presence of GSH and HAase at pH=5.0, thus playing an anti-tumor and bacteriostatic role.

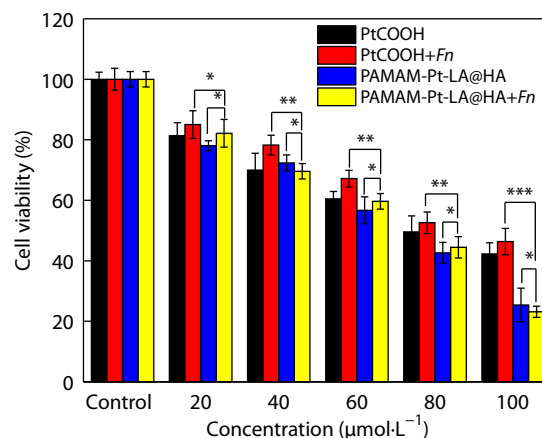


Fig. 9 Cell viability of HCT116 cells under treatment of PBS (Control), PtCOOH, PtCOOH + *Fn*, PAMAM-Pt-LA@HA and PAMAM-Pt-LA@HA + *Fn* at different platinum concentrations. Mean $\pm$ SD, $n=3$. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

CONCLUSIONS

To conclude, we synthesized a new dendrimer-based nanocomplex with anti-tumor and antibacterial dual-functions to against the harmful bacterial induced drug resistance in the treatment of CRC. The nanocomplex was constructed through conjugating Pt(IV)-complex and LA to the dendritic carrier PAMAM ($G=3$), and further coated with HA on its surface. The exposed HA could either enhance uptake of tumor cells or reduce cytotoxicity to normal cells, and thus could realize targeting delivery to tumor tissues. Moreover, the hyaluronidase produced by *Fn* or in the tumor cells could decompose the coated HA and thus the Pt(IV)-complex and LA could be released from the nanocomplex to exhibit antitumor and antibacterial activities. This dual-functional nanocomplex provided a new method to develop anticancer nanomedicines with more therapeutically efficacious for the treatment of CRC. This strategy that through against the harmful bacterial existed in tumor tissues to enhance the antitumor activities of anticancer drugs also provided new insight to design anticancer nanomedicines for the treatment of other harmful bacterial co-existed cancers, such as breast cancer and pancreatic ductal carcinoma.

NOTES

The authors declare no competing financial interest.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://doi.org/10.1007/s10118-023-2890-x>.

ACKNOWLEDGMENTS

This work was financially supported by the National Natural Science Foundation of China (Nos. 22075209, U20A20260 and 52103180); a distinguished professor of Tianjin, the Leading

Talents Program of the Tianjin Educational Committee; the Key program of Tianjin Municipal Natural Science Foundation (Nos. 18JCZDJC37700 and 18JCQNJC72300); the Training Project of Innovation Team of Colleges and Universities in Tianjin (No. TD13-5020); and the Tianjin Enterprise Key Laboratory for Application Research of Hyaluronic Acid (No. KTRDHA-Z201901) for the financial support. We would like to thank the Analytical & Testing Center of Tiangong University for Thermal Field-Emission Scanning Electron Microscope work.

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