Hyaluronic acid modified micelles encapsulating Gem-C_{12} and HNK for glioblastoma multiforme chemotherapy

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1 Materials

Hyaluronate sodium (molecular weight of 89 kDa) was purchased from Bloomage freda biopharm Co., Ltd (Jinan, China). Other reagents and chemicals were analytical grade and purchased commercially.

Male Sprague–Dawley rats and Balb/c nude mice were obtained from Institute of Laboratory Animals of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital (Chengdu, China). All animal studies presented were performed per the institutional guidelines and approved by the Ethics Committee of Sichuan University.

2 Methods

2.1 Synthesis of HA-deoxycholic acid copolymer

HA-deoxycholic acid copolymer (HA-DA copolymer) was synthesized following the published method [1,2] (Fig. S1). Briefly, methyl deoxycholate was prepared by dissolving DA (5 g, 12.5 mM) in methanol (30 mL), then 0.6 mL H_2SO_4 was added, and the mixture was heated to reflux for 1 h with magnetic stirring. Then the reaction solution was cooled down in ice cold water, the crystal was collected and washed twice with cold methanol. Then deoxycholyl ethylenediamine (DCEA) was obtained by transesterification reaction. Methyl deoxycholate (2 g, 5.9 mM) was dissolved in ethylenediamine (10 mL) and the mixture was heated to reflux for 5 h. After the reaction finished and cooled down, the solution was poured into the ice cold water, and the precipitation was collected, washed, dried in vacuum and recrystallize in methanol and ethyl acetate (1:10). HRMS (ESI) of both methyl deoxycholate and DCEA were recorded on a LC-QTOF (maXis II, Bruker Co., USA). Finally, the HA-DA copolymer was synthesize by coupling DCEA and HA in methanamide and dimethyl formamide solution (1:1) with the catalyzing of 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC.HCl) and 1-Hydroxybenzotriazole (HOBt). The reaction was lasted for 48 h, then the mixture was dialyzed for another 48 h, and the white solid product was obtained by lyophilization.

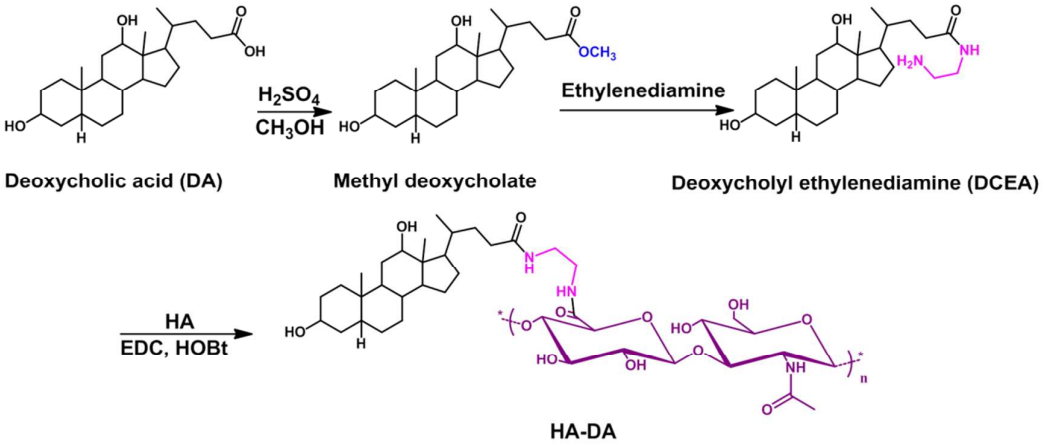

Fig. S1. Synthetic scheme of HA-DA copolymer.

2.2 Drug release in vitro

The in vitro release of Gem-C_{12} and HNK from micelles was measured using the dialysis setup [3]. Briefly, 1.0 mL of plain-M, HA-M suspensions or Gem-C_{12} & HNK solution (20% ethanol in PBS) were placed into dialysis bags (MWCO 3.5 kDa), respectively. Then the dialysis bags were immersed into 100 mL of phosphate buffer solution with 0.5% tween 80 (w/v) (PBS, pH 7.4) and incubated at 37 °C under constant shaking at 100 rpm. At the predetermined time intervals, 1.0 mL of release medium was collected and replaced with 1.0 mL of fresh medium. The amount of Gem-C_{12} and HNK released were measured using HPLC.

2.3 Analysis of CD44 expression

To characterize the expression of CD44 on U87 cell surface, flow cytometry assay was performed as previously described with slight modification [4]. Briefly, 5×10^5 U87 cells were cultured, collected and stained with CD44-FITC (Invitrogen, Thermo Fisher Scientific) antibody according to the manufacturer's instructions. B16F10 cells was used as positive control, which were reported as CD44 high expressing cells and HK2 cells were used as negative control [3,5]. The fluorescent intensity of cells was measured by flow cytometer (Cytomics^{TM} FC500, Beckman Coulter, USA).

2.4 In vivo brain distribution in a brain injury model

To confirm whether the intracranial injection technique would disrupt the BBB, a brain injury model was established by intracranially injection of 5 μL of PBS into the right hemisphere. Two weeks later, the mice were divided into three groups (n = 3) and treated with PBS, free DiD, DiD-M and DiD-HA-M via the tail vein at a dose of 200 μg/kg, respectively. At 4 h post-injection, the mice were anesthetized with i.p. administered 4% chloral hydrate, fixed by heart perfusion with saline and 4% paraformaldehyde. Then, the glioma-bearing brains were excised, washed and the fluorescence was imaged through an in vivo imaging system (PE IVIS Lumina, USA). Then, they were fixed in paraformaldehyde for 48 h, dehydrated by 15% sucrose solution and 30% sucrose solution for 24 h sequentially until subsidence. Afterward, they were sliced (10 μm) and stained with DAPI (1μg/mL) and visualized by a confocal microscope (FV1200, Olympus, JPN).

2.5 Qualitatively pharmacokinetic research

To qualitatively research the in vivo behavior of plain-M and HA-M, micelles were labeled with DiD for in vivo imaging system detection. Brifly, SD rats (180–200 g) were divided into three groups randomly (n = 3). DiD labeled plain micelles (DiD-M) and HA-M (DiD-HA-M) were administered intravenously (DiD dose was 200 μg/kg). At the scheduled time points (5, 15, 30, 60, 120, 240, 360, 480, 600 and 720 min) after injection, 200 μL of blood were collected in eppendorf tube, and DiD fluorescence images were scanned by an in vivo imaging system (PE IVIS Lumina, USA).

3 Results

3.1 Synthesis and characterization of HA-DA

DA has been widely used as a hydrophobic moiety to synthesize amphiphilic materials [6,7]. The HA-DA copolymer was synthesized using ethylenediamine as a linker to HA and DA. The structures of methyl deoxycholate and DCEA were confirmed by high resolution mass spectrometer (HRMS, ESI). The mass-to-charge

ratio for methyl deoxycholate was m/z 429.2956 $[M + Na]^+$ (calcd. for $C_{25}H_{42}O_4$ 406.3083), and for DCEA was m/z 435.3566 $[M + H]^+$ (calcd. for $C_{26}H_{46}N_2O_3$ 434.3508).

The HA-DA copolymer was characterized by ^{1}H NMR. As shown in Fig. S2A the characteristic peaks of HA appeared at 2.00 ppm (-$COCH_3$, a) and 3.30 - 4.80 ppm (sugar ring, b). As for HA-DA, the additional characteristic peaks of DA (0.20 - 1.50 ppm, c) indicated the successful synthesis of HA-DA [8]. The amount of deoxycholic acid in HA-DA was quantitatively characterized using the integration ratio between the characteristic peaks of the *N*-acetyl group in HA (2.00 ppm, 3H, -$COCH_3$-) and the methyl group in deoxycholic acid (0.80 ppm, 3H, -CH_3). The substituting degree of HA-DOCA was about 9.5% (Fig. S2B).

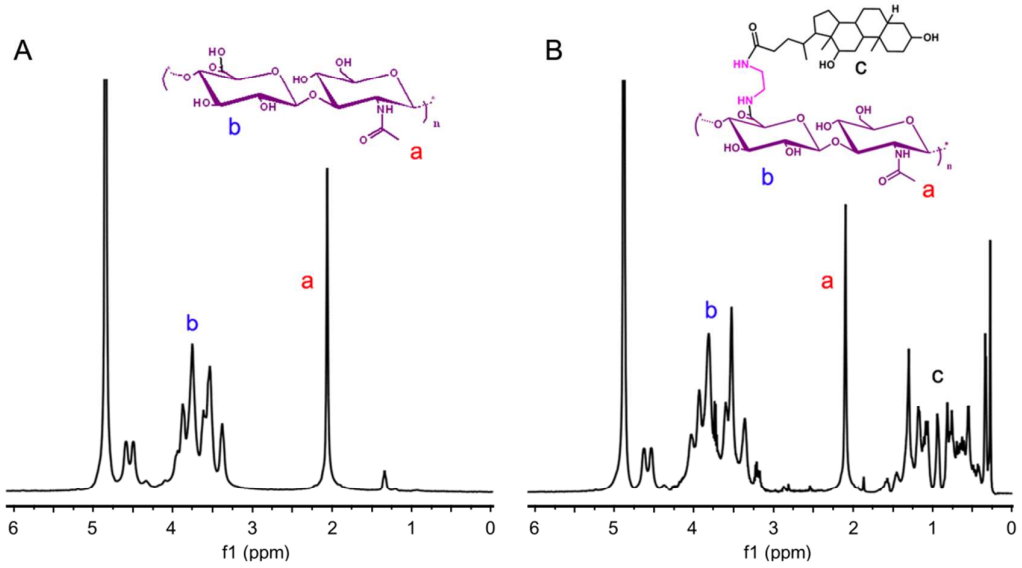

Fig. S2. ^{1}H NMR spectra of HA (A) and HA-DA copolymer (B).

3.2 In vitro release

HNK showed a faster release behavior with the 24 h cumulative release of 56.5% and 48.87% for plain-M and HA-M, while 94.8% for free drug solution (Fig. S3). However, the release rate of Gem-C_{12} was slower, only 79% was released from Gem-C_{12} & HNK solution after 24 h incubation. After loaded into micelles its release was further restricted, with the 24 h cumulative release of 42.4% by plain-M and 39.5% by HA-M. In a period of 4 d, 87% and 73% of HNK in plain-M and HA-M can be

released, while only 63% of Gem-C_{12} can be detected in the medium. The HA modification could delay the release of HNK, however, Gem-C_{12} release profile was not changed in this method.

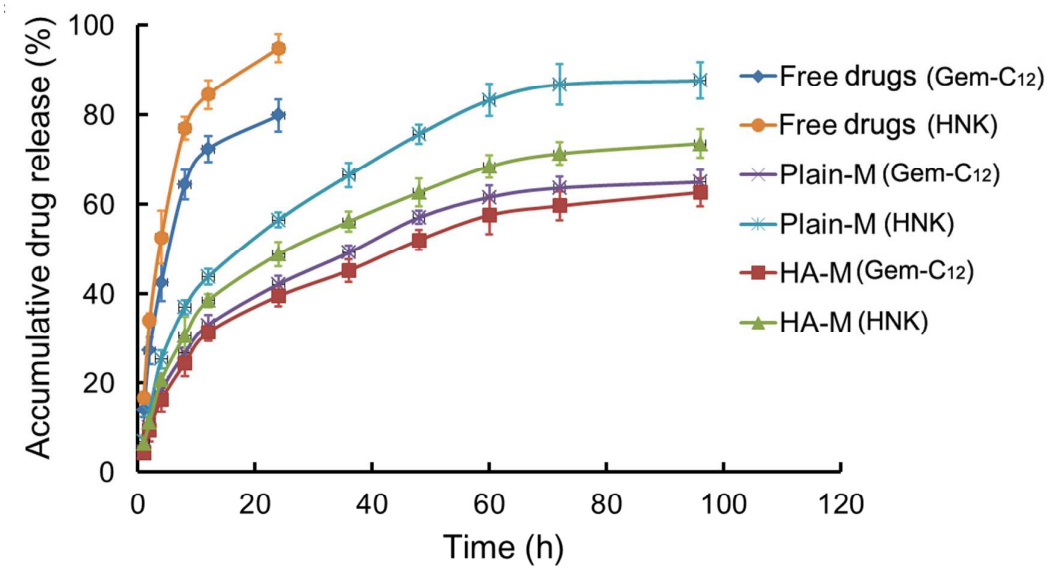

Fig. S3 In vitro release profiles of Gem-C_{12} and HNK from free Gem-C_{12} & HNK solution, plain-M and HA-M in PBS at pH 7.4 containing 0.5% Tween 80 (w/v). Data represents mean ± S.D. (n = 3).

3.3 Analysis of CD44 expression

The expression of CD44 in U87 cells was examined by flow cytometry after immunohistochemistry staining. As shown in Fig. S4, a similarly high CD44 expression was detected both on U87 cells and B16F10 cells, however no CD44 was detected on HK2 cells. The high expression of CD44 in U87 cell lines enable CD44 endocytosis of HA-grafted micelles into glioma cells.

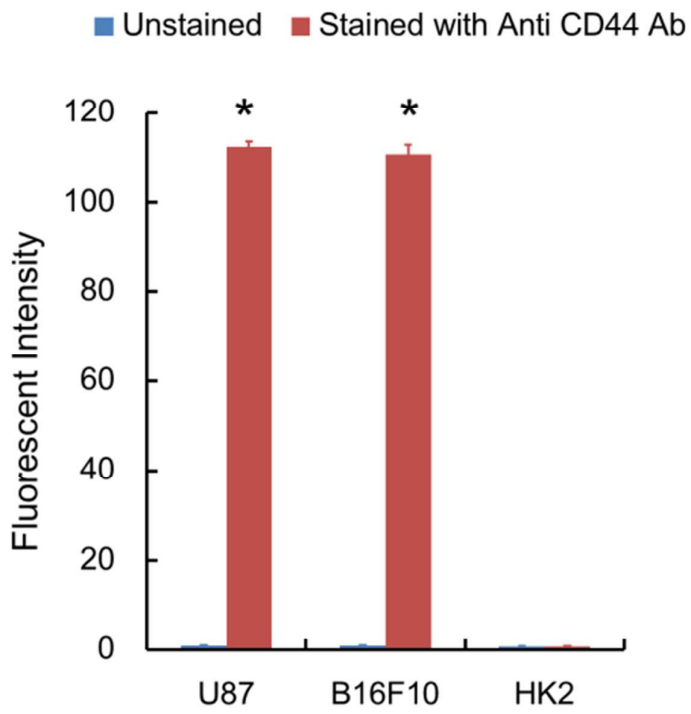

Fig. S4 Fluorescence intensity of unstained and stained cells (U87, B16F10 and HK2) measured by flow cytometry. Data represents mean ± S.D. (n = 3). * $p < 0.01$ compared with HK2.

3.4 In vivo brain distribution in a brain injury model

As Fig S5 showed that no fluorescence signal was detected in the brain slices, which confirmed that the intracranial injection technique would not disrupt the BBB and make the accumulation of micelles at the injection site. This result was consistent with the findings by Eun Ji Chung en al [9].

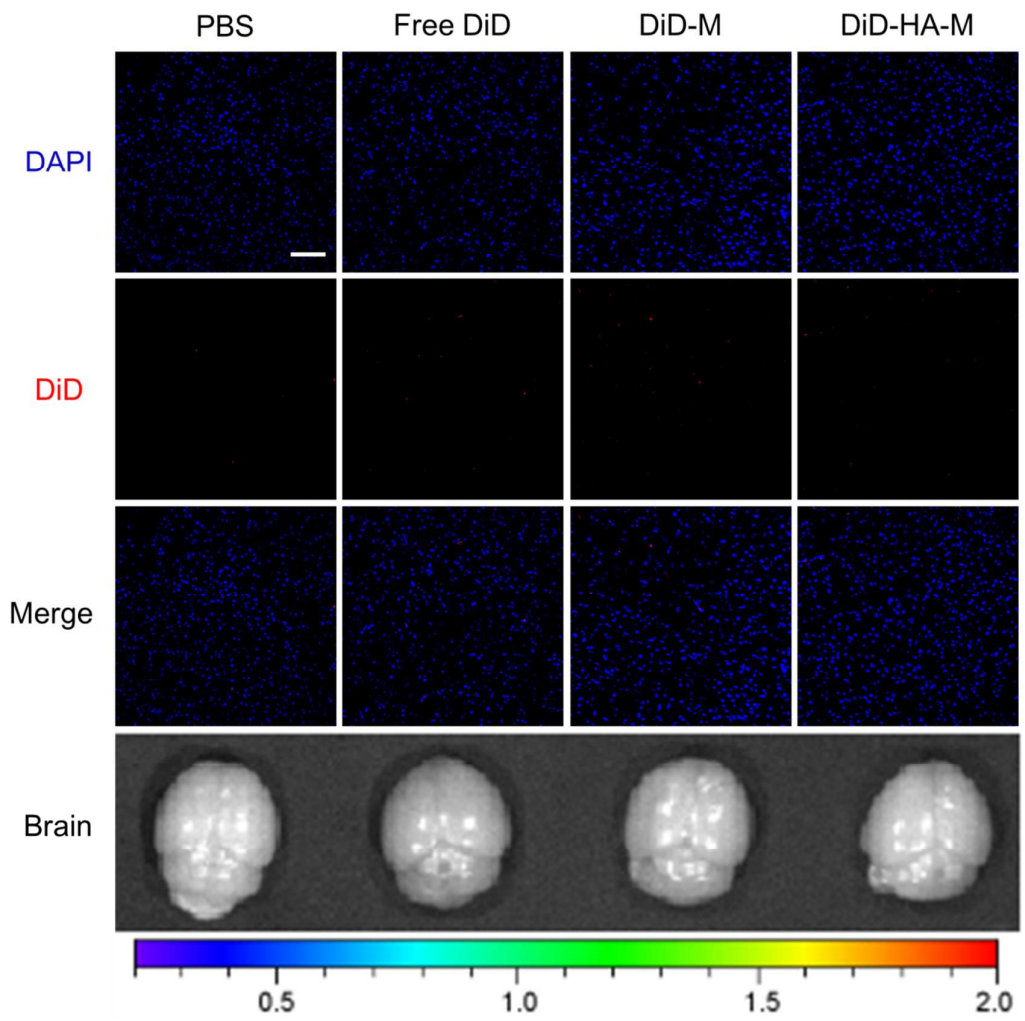

Fig. S5 Ex in vivo fluorescence image of the brain slices and brains. No fluorescence signal was detected in the brain injury model. Blue: cell nuclei. Red: DiD. The scale bar represents 100 μm.

3.5 Qualitatively pharmacokinetic research

The blood concentration of plain-M and HA-M were represented by the DiD fluorescence intensity of blood samples. As the shown in Fig. S5, blood samples treated with DiD-M and DiD-HA-M have much stronger fluorescence intensity compared to that of free DiD, which meant a prolonged blood circulation time. Moreover, the stronger fluorescence intensity was detected by injection of DiD-HA-M at the initial 60 min, which indicated a better stability of HA-M than that of plain-M in the blood circulation.

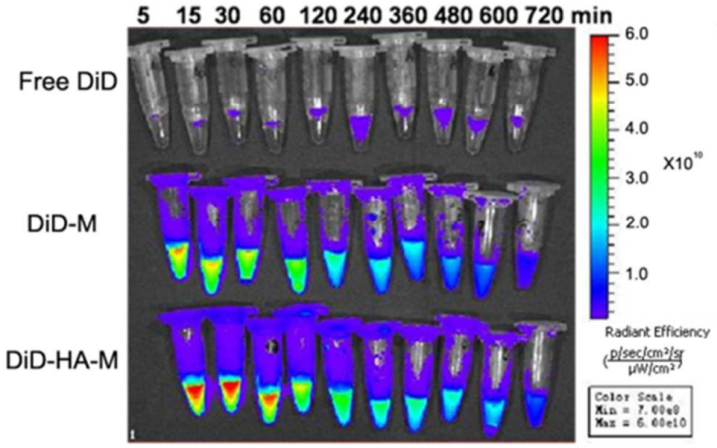

Fig. S6 Ex vivo fluorescence image of plasma in different groups treated with free DiD, DiD-M and DiD-HA-M at the predetermined time points.

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