Supporting information

Enhanced transdermal lymphatic drug delivery of hyaluronic acid modified transfersome for tumor metastasis therapy

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Materials

Sodium form of hyaluronic acid (HA, MW, 10 kDa) was a gift of Kolon Life Science, Korea. EDC.HCl (99%) and N-Hydroxy succinimide (NHS, 99%) were purchased from Shanghai Medeep Co., Ltd. Monostearin (glycerol α-monostearate, GMS), Sodium deoxycholate (DOC) and fluorescein isothiocyanate (FITC) were purchased from Solarbio (Beijing, China). Doxorubicin hydrochloride (DOX) was supplied by Zhejiang Hai zheng Co. Ltd. (China). All other reagents and solvents were of analytical grade.

Preparation and characterization of hyaluronic acid modified transfersome (HA-GMS-T)

Amphiphilic HA-GMS were synthesized using a previous method.[1] HA-GMS was labeled by FITC or rhodamine for fluorescence identification, respectively.[1,2] Transfersomes were prepared through lipid film method. Briefly, 70 mg lecithin was dissolved in chloroform/ethanol (1:1, v/v) and evaporated to dry to form lipid film under reduced pressure. Then, the transfersomes were produced by hydration of the lipid film with 10 mM PBS (pH 7.4) containing 25 mg DOC at 55 ℃ for 30 min, and size reduction with sonication. HA-GMS was added during hydration with final concentration of 1 mg/mL to give HA modified transfersome (HA-GMS-T). Various parameters including lecition/DOC ratio, hydration temperature, duration and medium, pH condition and solvent were screened out to optimize the preparation (Table S1).

Size analysis and zeta potential were determined on a Zetasizer ZEN 3600 Nano Series apparatus (ZEN, UK). The morphology of HA-GMS-T was observed via TEM (JEM-1200EX JEOL Ltd., Japan) and CLSM (Carl-Zeiss, German) at Ex 543 nm. Transmittance at 600 nm of HA-GMS-T was determined to evaluate its stability.

Table S1 Transmittance, particle size and PdI of HA-GMS-T prepared under various conditions (n=3).

Lecithin/DOC（w/w）	T/℃	Hydration time /h	Sonication /times	hydration medium	pH	Solvent （v/v）	Transmittance /T%	Particle size/nm	PdI
70/20	55	0.5	4*20 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	81.5±4.72	98.65±0.45	0.42±0.02
70/25	55	0.5	4*20 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	92.0±1.89	91.74±3.07	0.42±0.01
70/30	55	0.5	4*20 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	95.7±4.21	99.62±3.25	0.43±0.01
70/25	35	0.5	4*20 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	95.5±3.68	101.61±1.38	0.44±0.01
70/25	55	0.5	4*20 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	92.0±1.89	91.74±3.07	0.42±0.01
70/25	70	0.5	4*20 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	92.5±2.06	121.72±0.54	0.48±0.02
70/25	55	0.5	4*20 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	92.0±1.89	91.74±3.07	0.42±0.01
70/25	55	2	4*20 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	95.4±1.63	146.91±3.85	0.50±0.01
70/25	55	4	4*20 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	94.4±2.65	146.85±4.82	0.49±0.02
70/25	55	0.5	0 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	94.6±4.30	101.33±3.75	0.43±0.04
70/25	55	0.5	4*20 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	92.0±1.89	91.74±3.07	0.42±0.01
70/25	55	0.5	6*20 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	95.7±2.83	100.74±5.60	0.42±0.02
70/25	55	0.5	4*20 s	water	7.4	ET:$CHCl_3$=1:1	81.0±3.03	99.18±2.74	0.37±0.01
70/25	55	0.5	4*20 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	92.0±1.89	91.74±3.07	0.42±0.01
70/25	55	0.5	4*20 s	100mM PBS	7.4	ET:$CHCl_3$=1:1	15.6±0.84	100.48±4.27	0.37±0.01
70/25	55	0.5	4*20 s	10mM PBS	6.5	ET:$CHCl_3$=1:1	29.1±1.62	99.51±5.80	0.34±0.02
70/25	55	0.5	4*20 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	92.0±1.89	91.74±3.07	0.42±0.01
70/25	55	0.5	4*20 s	10mM PBS	8.5	ET:$CHCl_3$=1:1	93.1±3.46	99.05±0.36	0.36±0.04
70/25	55	0.5	4*20 s	10mM PBS	7.4	ET	85.5±1.50	102.18±4.20	0.41±0.01
70/25	55	0.5	4*20 s	10mM PBS	7.4	ET:$CHCl_3$=1:1	92.0±1.89	91.74±3.07	0.42±0.01
70/25	55	0.5	4*20 s	10mM PBS	7.4	$CHCl_3$	95.8±4.01	101.55±3.74	0.42±0.02

Data represented the mean±SD, n=3.

Optimization of doxorubicin loaded HA-GMS-T (DOX-HA-GMS-T)

DOX-HA-GMS-T was prepared by adding DOX into hydration solvent with final concentration of 1 mg/mL. Response surface methodology (RSM) was used to optimize the formulation parameters for the encapsulation efficiency (EE %) and loading capacity (LC %) of DOX-HA-GMS-T. A 3-factor, 3-level design was used to explore the quadratic response surfaces and for constructing second order polynomial models using Design Expert® (Version 7.0.0, Stat-Ease Inc., Minneapolis, MN). The independent variables (Table S2), including the Mass of DOC (X_1), hydration temperature (X_2) and concentration of DOX (X_3) were defined in three levels (low, medium and high). 17 experiments including 12 factorial points with five replicates at the center point for estimation of pure error sum of squares were employed. DOX-HA-GMS-Ts were purified by Sephadex G25 fine column. The amount of loaded DOX was determined with FLx8000 Biotek plate reader (Biotek, Winooski, Vermont) at Ex/Em 537/584 nm.

Table S2 Experimental levels of the independent variables for the BBD

Variables	Factors	Code		
		-1	0	1
X_1	Mass of DOC (mg)	20	25	30
X_2	Temperature(℃)	45	55	65
X_3	Concentration of DOX (mg/mL)	0.5	1	1.5

Cytotoxicity and hemolysis of HA-GMS-T

(1) Cytotoxcity

Cytotoxicity of HA-GMS-T was studied by MTT assay using mouse embryo fibroblasts (MEFs). The primary culture of MEFs were seeded at 1×10^4 cells/well in 96-well plates and incubated at 37 ℃ for 24 h. Then, serious concentrations (0.0625, 0.125, 0.25, 0.5, 1 mg/mL) of HA-GMS-T in culture medium were incubated for 24, 48 and 72 h. The HA-GMS-T free culture was taken as control. The relative cell proliferation (RCP%) was defined as RCP% = (OD 490 sample/OD 490 control) × 100%.

(2) Hemolysis

Hemolysis assay was performed using citrated whole blood obtained from healthy male New Zealand white rabbit. Erythrocytes were separated by centrifugation, rinsed and resuspended with PBS (pH 7.4) to obatain 2% (w/w) erythrocytes stock dispersion. The approximate amount of red blood cell was 3.75×10^8 cells/mL. 1 mL HA-GMS-T of series concentrations were added to 0.5 mL

erythrocyte stock dispersion. After incubation for 1 h, unlysed erythrocytes and debris were removed by centrifugation (1500 × g, 10 min), and absorbance of the supernatant was measured at 540 nm on UV1200 UV/vis spectrophotometer (Mapada, China). The hemolysis elicited by the Triton X-100 solution (5%) was designated to be 100% while the hemolysis given by the PBS (pH 7.4) was taken as 0%. The hemolysis rate (HR%) was calculated by following equation:

HR %=(Dt-Dnc)/(Dpc-Dnc)×100%

where Dt, Dnc, and Dpc are the absorbances of the tested sample, the negative control, and the positive control, respectively.

In vitro skin permeation study of DOX-HA-GMS-T

(1) Skin preparation

Wistar rats were used (Male Wistar Rats weighting 200-250g, Qingdao Municipal Institute for Drug Control, China) were taken to study the transdermal penetration of HA-N. The study was conducted in accordance with the guidelines of University laboratory Animal ethical Committee. Hairs on dorsal skin were removed with lab-made hair removal solution a day before experiment. The mice were sacrificed by cervical dislocation. The full thickness dorsal skin was excised and subcutaneous tissues were carefully removed with surgical scissors.

(2) Skin permeation

Skin permeation studies were performed on modified static-type Franz cells (diameter 1.5 cm, volume 5 mL, Rightway Technology Co., Ltd, China). The skin tissue was placed between the donor and acceptor chambers of the diffusion cells with the epidermal side facing upwards. The excess skin was trimmed off and the acceptor chambers were filled with degassed PBS (pH 7.4). The acceptor phase was stirred magnetically to equilibrate for 30 min at 37.2 °C. 1 mL HA-GMS-T (HA-GMS 1 mg/mL) and naked transfersome loaded with DOX (1.54 mg/mL) were added to donors and the acceptor chambers were filled with fresh PBS (7.4). DOX solution (1.54 mg/mL) was taken as control group. 0.1 mL samples were withdrawn at intervals (1, 2, 3, 4, 6, 8, 10, 12 and 24 h) and replenished with fresh degassed PBS. The whole process of manipulation should avoid formation of bubbles. The samples were filtered through 0.45 mm membrane filter and analyzed for drug content by fluorescence plate reader. The cumulative active ingredient penetration (Qt, μg/cm^2) through the skin was calculated from the following equation:

$$Qt = VrCt + \sum_{i=0}^{t-1} VsCi$$

Ct is the drug concentration of the receiver solution at each sampling time, Ci is the drug concentration of the ith sample, and Vr andVs are the volumes of the receiver solution and the sample, respectively. Data were expressed as the cumulative drug permeation per unit of skin surface area, Qt/S. The steady-state fluxes (Jss, mg/cm^2·h) were calculated by linear regression interpolation of the experimental data at a steady state:

$$Jss = \Delta Qt / (\Delta t \times S)$$

(3) Fluorescence microscopic observation

Upon the full thickness skin tissue mounted on Franz cells, 1 mL FITC-HA-GMS-T (HA-GMS 1 mg/mL) or naked transfersome loaded with DOX (1.5 mg/mL) was added to the donor cells, leaving the permeation last for 4 h. The skin tissue was rinsed with PBS (7.4) and frozen at -20 ℃. Cryotome sections of 30 μm thickness (Thermo Scientific Cryotome FSE, Germany) were subjected to inverted fluorescence microscope (Nikon Eclipse Ti-S, Nikon Ltd, Japan) and the camera integration time was set to 3 s.

In vivo tissue distribution of DOX-HA-GMS-T

Wistar rats (male, 4 weeks age, average weight: 200g) were prepared. The rats were fasted overnight to prevent food fluorescence interference in GI tract. The rat dorsal surface hair was removed by a clipper. Lab-made non-occlusive containers (diameter 13 mm, volume 0.13 mL) were adhered on the hairless skin. The area of filter contact with skin is the effective area of transdermal drug delivery (1.33cm^2, diameter 13mm, thickness 10mm). The head of rat was covered to avoid bite and lick.[3] Rats were treated with DOX-T or DOX-HA-GMS-T (HA-GMS 1mg/mL, DOX 1.54 mg/mL) every 8 hours at dosage of 0.1 mL per time. Rats without treatment were taken as negative control. 24 hrs later, rats were sacrificed, and the fluorescence distribution of DOX in vivo was observed by small animal in vivo imaging instrument (Fusion FX7, Vilber Lourmat, France), with filter F595, exposure time 1 s and aperture 1.2. Major organs (heart, liver, spleen and kidneys) and cervical lymph nodes were dissected. Certain weight of each organ was homogenized in 2 mL tris-HCl (pH 7.4) using tissue homogenizer at 15,000 rpm for 5 min. The DOX fluorescent signal was detected by small animal in vivo imaging instrument and quantified with processing software (bio-1D Version 15.06a). The auto-fluorescence emitted by animal in negative control group was subtracted from the results.

Cellular uptake study

Firstly, due to the cytotoxicity of DOX, cellular internalizing capacity of DOX free HA-GMS-T was evaluated. Human breast cancer cell line MCF-7 were taken as model and cultured in DMEM (Gibco, Grand Island, NY, USA) containing FBS (10%) and penicillin-streptomycin (100 U/mL culture medium) at 37 °C in a 5% CO_2 humid atmosphere. After cell attachment, the medium was replaced by HBSS (pH 7.4) and equilibrated for 30 min. Fresh prepared HA-GMS-T (400, 200, 100, 50 μg/mL) was replenished, followed by incubation for 0.5 and 2 h, respectively. Cell samples without treatment were taken as negative control. The cells were then rinsed twice with PBS (pH 7.4) and treated by 0.5% Triton X-100 for 30 min to lyse the cells. The amount of endocytosed HA-GMS-Ts were measured by FITC fluorescence on plate reader (Ex/Em 485/528 nm). Calibration curve was constructed of FITC fluorescence intensity as function of HA-GMS-T with serial concentrations. Cellular uptake ratio (UR%) was calculated by

$$UR\% = 100 \times Cu/Ci$$

Hereinto, Cu and Ci were the uptake and initial concentrations of HA-GMS-T, respectively.

For cellular uptake of DOX loaded transfersome, DOX-HA-GMS-T and DOX-T

with HBSS (DOX: 620, 310, 160, 80 μg/mL) were replenished in cell culture and incubated for 2 h. The endocytosis of nanomedicine was observed by CLSM. Then, the cells were lysed and the cellular DOX levels were measured by DOX fluorescence (Ex/Em 537/584 nm) to calculate its uptake ratio.

Statistical analysis

All statistical evaluations were performed by OriginPro 8.0 (OriginLab Corporation, Northampton, MA, USA). Statistical significance was found by One-Way ANOVA, Tukey test was used to analyze differences. The presented data (arithmetic mean value ± standard deviation) resulted from at least three independent experiments ($n \geq 3$), the value of $p \leq 0.05$ was considered as significant.

References

[1] M. Kong, X. G. Chen and H. J. Park, *Carbohydr. Polym.,* 2011, 83, 462–469.
[2] M. Kong, H. J. Park, C. Feng, L. Hou, X. J. Cheng and X. G, Chen, *Carbohydr. Polym.,* 2013, 94(1), 634-641.
[3] Y. Y. Gao, X. J. Cheng, Z. G. Wang, J. Wang, T. T. Gao, P. Li, M. Kong, X. G. Chen, *Carbohydr. Polym.,* 2014, 112, 376-386.

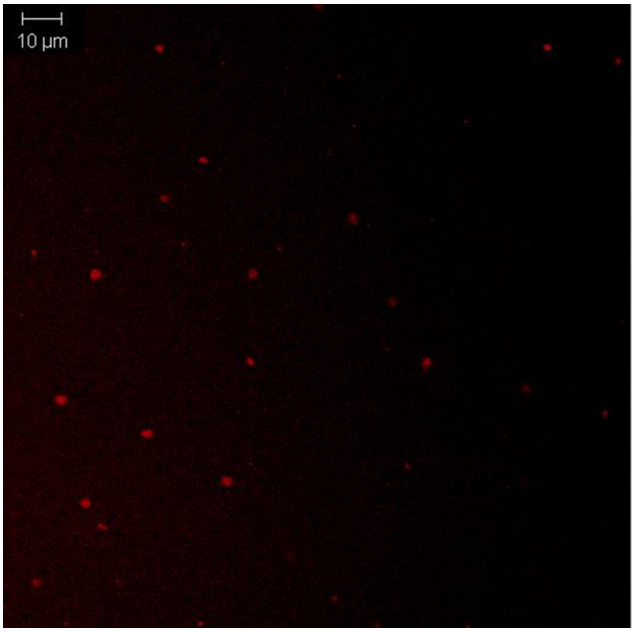

Fig. S1 CLSM images of doxorubicin labeled HA-GMS-T. Bars represent 10 µm.

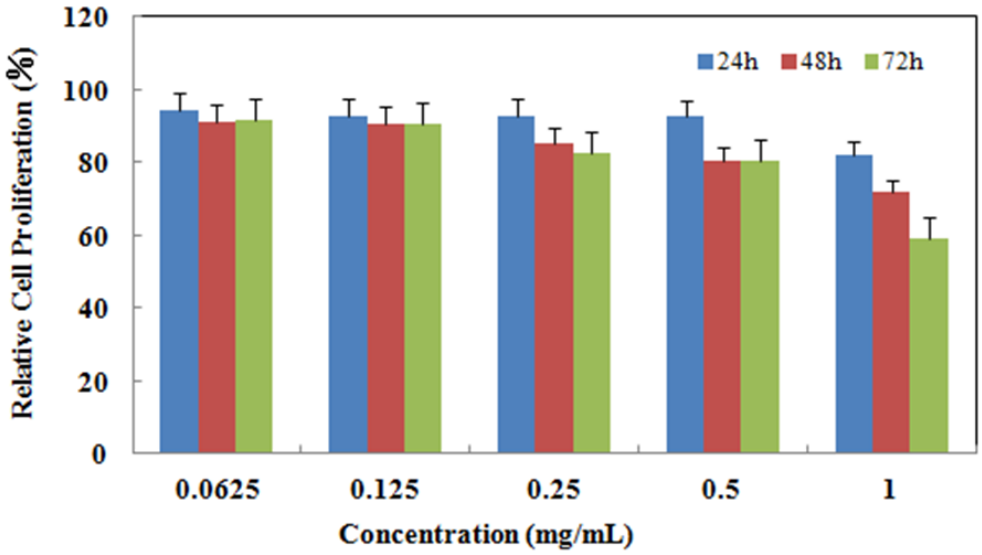

Fig. S2 Effects of HA-GMS-Ts on relative proliferation of MEF cells under various concentrations and incubation period.

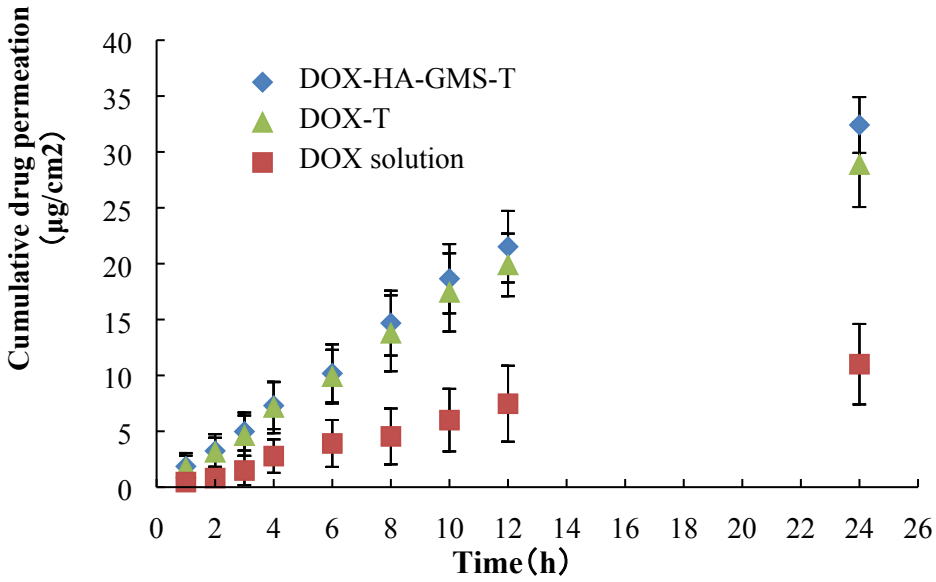

Fig. S3 In vitro permeation profiles of doxorubicin through the excised rat dorsal skin from DOX-HA-GMS-T and DOX-T (HA-GMS 1mg/mL, DOX 1.54 mg/mL). The control sample was solution of DOX (1.54 mg/mL) (Mean±S.D., n=5).