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Hyaluronic Acid Coated Liposome for Active Targeting on CD44 expressing tumors

Zhenfen Tian[1], Jianwen Liu[2], Na Li[3], Vasil M. Garamus[4], Aihua Zou*[1]

[1] Shanghai Key Laboratory of Functional Materials Chemistry, State Key Laboratory of Bioreactor Engineering and Institute of Applied Chemistry, School of Chemistry & Molecular Engineering, East China University of Science and Technology, Shanghai 200237, PR China

[2] State Key Laboratory of Bioreactor Engineering & Shanghai Key Laboratory of New Drug Design, School of Pharmacy, East China University of Science and Technology, Shanghai 200237, P. R. China

[3] National Center for Protein Science Shanghai and Shanghai Institute of Biochemistry and Cell Biology, Shanghai 200237, P. R. China

[4] Helmholtz-Zentrum Geesthacht, Centre for Materials and Coastal Research, D-21502 Geesthacht, Germany

*To whom correspondence should be addressed.

Tel/Fax.: +86-21-64252231

E-mail: aihuazou@ecust.edu.cn

†East China University of Science and Technology.

Abstract Liposome coated with hyaluronic acid (HA) was fabricated for targeted delivery of Doxorubicin hydrochloride (DOX) to CD44 expressing tumors. DOX was incorporated into liposome (DOX-L) *via* a transmembrane pH-gradient method, which contributed to high encapsulation efficiency (97%) and drug loading (19%). HA was modified on the surface of DOX-L by simple vortex (HA-DOX-L). Both DOX-L and HA-DOX-L had the average diameter around 110 nm with good uniformity and showed good stability during 6 months storage. SAXS and TEM evidenced the corona of HA on the surface of DOX-L, which convinced the prolonged circulation of DOX. The apoptosis study demonstrated the improved efficacy of HA-DOX-L with the human colon cancer cell line HCT-116 cells in comparison to the conventional reservoirs. This improved efficacy of HA-DOX-L with HCT-116 cells should be related with the interaction between HA and CD44 receptor of HCT-116 cells.

Keywords DOX;liposome; HA; CD44; target

Introduction

Since liposomes were suggested as pharmaceutical carriers in cancer chemotherapy by Gregoriadis et al. in 1974, the researches in liposomes kept increase due to their size control properties, biocompatibility and loading capacity with both hydrophilic and hydrophobic drugs(Rahman et al. 1974). While conventional liposomes suffered with the problems of non-specifical distribution in the body, easily caused dose-related side effects and the fast blood clearance by the reticuloendothelial system (RES), resulted in inadequate drug concentrations reaching the tumor cells(Andresen et al. 2005). To solve these problems, the surface modified targeted liposomes, especially active targeted liposomes, emerged as one of the smartest delivery systems which could prolong *in vivo* blood circulation times and pass barriers imposed by the biological environment up to target cells(Paliwal et al. 2011). Hyaluronic acid (HA) is a non-toxic, non-immunogenic and biodegradable biopolymer. Owing to its high affinity for the cell surface adhesion molecule CD44, which is overexpressed in many tumour cells, such as epithelial, ovarian, colon, stomach, and acute leukemia, HA and its derivate have been widely utilized as active-targeted moieties modified on liposomes to selectively diagnose and treat CD44 abundant cancers(Toole. 2004; Zöller. 2011; Kim et al. 2012; Csoka et al. 2001; Bourguignon et al. 2002). Ju-Hwan Park prepared amphiphilic hyaluronic acid derivative (hyaluronic acid–ceramide; HACE)-coated nanohybrid liposomes for targeted delivery of anticancer drug and MR imaging of cancer. The results of *in vitro* cellular uptake assay showed that the cellular uptake of DOX from the nanohybrid liposome was enhanced by HA and CD44 receptor interaction, versus the plain liposome(Park et al. 2014). Dan Peer linked HA to the surface of nano-liposomes by covalent modification. Mediated by targeted hyaluronan liposomes, DOX accumulation in tumor-bearing lungs was 30-, 6.7-, and 3.5-fold higher than free DOX, non-targeted liposomes, and Doxil, respectively(Peer et al. 2004). The results of above preparations in terms of cytotoxicity activity were very promising *in vitro* and *in vivo* studies. However, the chemical reactions involved in the combination of HA and liposomes were complicated, and the introduction of chemical reagents can cause toxicity.

In this paper, we used DOX as a drug model and was incorporated into liposome (DOX-L) *via* the pH-gradient method, which exhibited a noticeable advantage for drug encapsulation, and it could be performed immediately preceding administration, thus eliminating drug leakage during storage(Cullis et al. 1991). Then, HA was coated on the optimized DOX loaded liposome (HA-DOX-L) for active targeting to tumor cells by vortex, which avoided the use of organic solvent and was simple and

controllable. Dynamic light scattering (DLS), transmission electron microscope (TEM) and small angle X-ray scattering (SAXS) measurements were used for structure characterization of DOX-L and HA-DOX-L. *In vitro* cell assays, including cytotoxicity and cell uptake, were also investigated. It is expected that HA coating the outer surface of DOX-L will overcome the limitations of non-selective delivery to the tumor site, and the hydrophilic polymer coating could also increase the stability of DOX-L *via* the formation of a protective shell.

Experimental Section

Materials

Doxorubicin hydrochloride (DOX, Beijing Hua Feng AllianceBernstein Co. Ltd, China); Soybean lecithin (Taiwei Co.Ltd, China); Cholesterol (J&K SCIENTIFIC Ltd, China); Cloroform (shanghai Chemical Reagent Co.Ltd, China); Hyaluronic acid (M.W. 1.2 MDa, Junchuang Biotechnology Co. Ltd, China); citric acid (Shanghai Lingfeng Chemical Reagent Co. Ltd, China); sodium citric (Shanghai Lingfeng Chemical Reagent Co. Ltd, China); freshly prepared ultra purified water; All other chemicals were of analytical grade or better.

Preparation of DOX-L and HA-DOX-L

The DOX-L preparation can be accomplished by two steps: blank liposome (Blank-L) and drug loading. Blank-L was prepared by film technique. Soybean phosphatidylcholine (SPC) and Cholesterol (CHOL), with a certain ratio (4:1, w/w), were dissolved in cloroform in a round-bottom flask. The cloroform was removed completely using a rotary evaporator (Yarong, Shanghai, China) under reduced pressure to form a thin film over the wall of the flask. The lipid films were subsequently placed under vacuum for a minimum of 3 h to remove any residual solvent. The inner aqueous solution at pH 3.8, citrate buffer, was then added to the dried film, followed by ultrasound until the entire film was suspended. The crude liposomes were then homogenized using the high pressure homogenization at 600 bar for 5 cycles to obtain the Blank-L.

The second step was to load DOX by pH-gradient method. The exterior pH of blank liposome was titrated with 1.0 M NaOH until a ΔpH between the inner and outer of liposomes was reached 4.0. DOX solution was added to the liposome with different DOX/lipid ratio (w/w)(Montero et al. 1993; Nakamura et al. 2012). Samples were heated at 60 °C for 60 min under continuously shaking.

As a vehicle for DOX-L and HA combination, HA at an appropriate concentration in sterile distilled water was made and then heated at 37 °C for 24 h in order to swell and dissolve completely. Then HA gel and liposome suspension were mixed with certain ratio on volume basis by vortex mixer(Dong et al. 2013).

Entrapment efficiency (EE) and drug loading (DL)

The encapsulation efficiency of DOX in liposomes was determined by ultrafiltration method. In brief, 400 μL of liposome dispersion was placed in the upper chamber of a centrifuge tube matched with an ultrafilter (MWCO10 kDa; Pall Corp, Port Washington, NY) and centrifuged at 10000 rpm for 30 minutes. The aqueous dispersion medium containing unloaded DOX was moved to the sample recovery chamber through the filter membrane. After separation, the amount of free DOX (W_{free}) was determined by UV–visible spectrophotometer (UV-1800, Shimadzu, Japan) at a wavelength of 495 nm. Regression equation and linearity (R^2) were $y=0.0193x-6.91393\times10^{-4}$ and 0.9994, respectively. The EE and DL were calculated by using the following equations(Li et al. 2009).

$$DL\ (\%) = (W_{total}-W_{free})\,/W_{lipid} \times 100 \quad (1)$$

$$EE\ (\%) = (W_{total}-W_{free})\,/W_{total} \times 100 \quad (2)$$

where W_{lipid} represented the amount of lipids, W_{total} indicated the total amount of drug added and W_{free} indicated the amount of drug un-encapsulated, respectively.

Particle size and polydispersity index

The mean particle diameter and polydispersity index (PDI) were measured by dynamic light scattering (DLS) using a Zetasizer 3000HSA (Delsa Nano C, Malvern Instruments, Malvern, UK) at 25 °C. All measurements were performed in triplicate.

Transmission Electron Microscopy (TEM)

The morphology of DOX-L and HA-DOX-L were examined by transmission electron microscopy (TEM) (JEOL-1400, Jeol, Tokyo, Japan). Before analysis, samples were diluted with distilled water and placed on a Formvar-copper grid (Science Services, Munchen), dried at room temperature, and then negatively stained with the aqueous solution of sodium phosphotungestic acid 2% (w/v).

In vitro drug release

In vitro release profile of DOX from DOX-L and HA-DOX-L was examined at 37 °C in release medium (phosphate-buffered saline, pH 7.4) for 3 days under protection from light. Briefly, 1 mL of DOX-L or HA-DOX-L were placed into a pre-swelled dialysis bag (MW cutoff = 8000–14,000). The dialysis bag was placed in 150 mL of release medium and gently shaken in a thermostatted shaker bath at 37 °C, 50 rpm. Samples were removed at appropriate intervals and replaced with the same volume of fresh buffer. The DOX released from liposome was assayed by UV–visible spectrophotometer (UV-1800, Shimadzu, Japan) at a wavelength of 495 nm. The accumulative release percentage (Q%) was calculated as follows(Zhang et al. 2007), and all experiments were carried out in triplicate:

$$Q\% = (C_n \cdot V + V_i \sum_{i=0}^{n-1} C_i) \Big/ (W_{drug\ \text{loaded liposome}} \times DL\%) \times 100\% \quad (3)$$

where C_n and C_i are the real time concentration of the removed samples at the time n and i respectively. V is the total volume of release medium, V_i is the volume of the removed samples at the time i (both V_0 and C_0 were equal to zero).

Small angle X-ray scattering (SAXS)

SAXS experiments were performed at beamline BL19U2 of National Center for Protein Science Shanghai (NCPSS) at Shanghai Synchrotron Radiation Facility (SSRF). The wavelength, λ of x-ray radiation was set as 1.033 Å. Scattered x-ray intensities were measured using a Pilatus 1M detector (DECTRIS Ltd). The sample-to-detector distance was set such that the detecting range of momentum transfer $q=4\pi\sin\theta/\lambda$, where 2θ was the scattering angle of SAXS experiments was 0.01-0.5 $Å^{-1}$. To reduce the radiation damage, a flow cell made of a cylindrical quartz capillary with a diameter of 1.5 mm and a wall thickness of 10 μm was used and the exposure time was set to 1-2 seconds. The x-ray beam with size

of 0.40×0.15 (H×V) mm^2, was adjusted to pass through the centers of the capillaries for every measurement. In order to obtain good signal-to-noise ratios, twenty images were taken for each sample and buffer. The 2-D scattering images were converted to 1-D SAXS curves through azimuthally averaging after solid angle correction and then normalizing with the intensity of the transmitted x-ray beam, using the software package BioXTAS RAW(Nielsen et al. 2009).

Cell lines and cell cultures

HCT-116 cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Science (Shanghai, China). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium and supplemented with 10% heat-inactivated bovine serum (Hyclone Laboratories Inc., Australia), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Gibco, Paisley, UK). Cells were maintained in an incubator at 37 °C with 95% air and 5% CO_2. When cells reached confluence, trypsin/ethylenediaminetetraacetic acid solution was used to split.

In vitro growth inhibition activity study

The cytotoxicity of free DOX, DOX-L and HA-DOX-L were determined by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay at the desired time. The HCT-116 cells (5×10^4) were seeded into 96-well culture plates and preincubated for 24 h. After that, the cells were treated with indicating concentration of pure DOX, DOX-L and HA-DOX-L, respectively. 48 h later, 20 μL MTT solution (5 mg/mL) was added into each well and incubated for 4 h at 37 ℃. The formazan crystals were then solubilized with DMSO. The absorption intensity was measured at 570 nm by an enzyme-linked immunosorbent assay reader.

Intracellular distribution of DOX-liposome and HA-DOX-liposome

To observe the intracellular distribution of DOX-L and HA-DOX-L in cancer cells, HCT-116 cells (1×10^5 cells per well in a 12 well plate) were exposed to DOX-L and HA-DOX-L (20 μg/mL DOX) for 6 h. After 6 h of incubation, the medium was removed, and the cells were washed with PBS followed by fixing with 4% paraformaldehyde in PBS for 10 min. Fluorescent images of cells were examined by

confocal microscopy (Carl Zeiss LSM510, Germany) and samples visualized using the 488 nm excitation of argon laser for fluorescein and 561 nm excitation of He-Ne laser for DOX.

Results and discussion

Formulation optimization and structural characterization of DOX-L and HA-DOX-L

DOX-L was prepared by pH-gradient method where the drug molecule can be loaded into the liposome by taking advantage of the pH-gradient between inner and outer side of the liposome(Nakamura et al. 2012). The effect of different DOX/lipid ratio on the physiochemical properties was shown in Table 1. The EE values of DOX-L almost kept constant with the increase of DOX/lipid ratio, until decreased to 87.62% when the ratio of DOX/lipid reached 1:4. While the values of liposome size, DL, and PDI of DOX-L increased all the time. High drug loading is a vital factor in liposome preparation(Zhang et al. 2014). From Table 1, samples 4 and 5 both obviously had higher DL than others. While compared with sample 5 (Table 1), sample 4 had a higher EE, smaller size and PDI. Thus, DOX-L with the DOX/lipid ratio at 1:5 ratio was chosen for further investigations.

HA was further combined with sample 4 to form HA-DOX-L. The average size and PDI values of HA-DOX-L were (117.2 ± 5.0) nm and 0.24 ± 0.02, respectively, i.e., slightly increased comparing with DOX-L (sample 4). Uniform size distributions for both DOX-L and HA-DOX-L can be seen in Fig.1. Fig. 1a and 1c showed that both formulations had narrow distributions and seemed to be generally homogeneous, which were consistent with their PDI values. The diameter of HA-DOX-L was slightly bigger than that of DOX-L due to the decoration of HA on the surface of DOX-L. The performed TEM investigation evidenced the existence of spherical liposome for both DOX-L and HA-DOX-L (Fig. 1 b, d, e). The sizes of some DOX-L and HA-DOX-L were around 50 nm from TEM images. And a particulate structure was observed for HA-DOX-L from the arrow in Fig. 1e, suggesting the coating of HA on the surface of liposome(Anabousi et al. 2005; Silvia et al. 2013). The sizes for DOX-L and HA-DOX-L determined by TEM measurements were obviously smaller than that obtained by DLS, which could be ascribed to the shrinkage of liposome after being dried in the sample preparation process for the TEM measurement.The sizes obtained by TEM were for the dehydrated liposome, while the sizes determined by DLS were their hydrodynamic diameter(Wu et al. 2014).

SAXS was used to get the mean or global features of the samples since TEM can image only a small part of the bulk volume. Fig. 2 represented SAXS curves for blank liposome (blank-L), DOX-L and HA-DOX-L samples. It should be noted that the characteristic scattering from a monodisperse system of spherical shells, could only give notable bumps on the curve related to the radius of the vesicles. In polydisperse systems, such bumps were smeared to a smooth decaying curve as a result of the superposition of many different liposome sizes. From Fig. 2, it can be seen that blank liposome showed a sharp maximum around 0.1 $Å^{-1}$. This was attributed to multilayer orderly structures to some liposomes in the system with mean distance between layers $2\pi/q_{max} \sim 60$ Å. After loading with DOX and further addition of HA, the position of maximum did not change (distance between layers was constant) but width of maximum of DOX-L, and HA-DOX-L increased, which pointed on the change of the number of layers in liposomes, and proved the incorporation of DOX and HA in liposome structures. Moreover, peak width is inverse proportional to the number of layers. Therefore, addition of DOX and HA leaded to the decrease of the number of layers in those multilayer liposomes. From Fig. 1b, some multilameller vesicle can be found for DOX-L, while most of these multilameller vesicles disappeared after the addition of HA. Hence, both SAXS and TEM results were well coincident with each other.

Encapsulation efficiency and drug release from liposomes

Fig.3 showed the encapsulation efficiency and drug loading for HA-DOX-L and DOX-L, which were around 98.21% 、 97.54% and 19.64%、 19.51%, respectively. Such high EE and DL values were attributable to pH-gradient method used in this study. Stability of liposomes (size, PDI, EE and DL) was evaluated with time period of 6 months in the storage condition at 4 °C (Fig.3). During the storage, no significant size change (lower than 10% for both DOX-L and HA-DOX-L) and no precipitation or liposome aggregation was observed for these two formulations. Furthermore, their EE and DL values were maintained around 90% and 19%. Accordingly, both DOX-L and HA-DOX-L were stable in the storage conditions for at least 6 months.

The results of *in vitro* release of DOX from DOX-L and HA-DOX-L were graphically represented in Fig. 4 It can be seen that the DOX release profiles displayed a sustained release without burst effect for both DOX-L and HA-DOX-L. This profile illustrated the good stability of DOX loaded liposome in PBS(pH = 7.4), which might prolong the drug release during the delivery process and enable DOX loaded liposome to accumulate and achieve efficacy in tumor tissue(Fan et al. 2013). Furthermore, HA-DOX-L

showed slower release than DOX-L. The cumulative amount of DOX released from HA-DOX-L over 70 h was 70%, while it was about 90% for DOX-L. That means the coating of HA on the liposome increased the thickness of liposome layer, and lead to the increase of the diffusion distance.

Biological evaluation in cell culture experiments

The expression of CD44 on the surface of HCT-116 cells was 89.15% which meant that CD44 was overexpressed in HCT-116 cells(Yang et al. 2013). To evaluate the anti-proliferative activity of DOX-L and HA-DOX-L to HCT-116 cells, the growth of HCT-116 cells was determined by MTT assay. As controls, free DOX, blank liposome and HA coated blank liposome (HA-L) were also prepared and applied to cells with the same procedures. As shown in Fig. 5, Blank-L and HA-L showed no obvious cytotoxicity to HCT-116 cells at equimolar lipid concentrations to DOX-L and HA-DOX-L. Thus, the cause of cells death with DOX-L and HA-DOX-L would be primarily from the effect of DOX loaded in liposome but not the materials of liposome. In case of free DOX, it was efficacious in inhibiting HCT-116 cells growth. There was only about 2.72% of cell viability at relatively low DOX concentration (1.25 μg/mL). This result was obviously more effective than DOX-L and HA-DOX-L, which still showed 39.3% and 30.5% cell viability at the same DOX concentration, respectively. While for free DOX, there was no significant improvement in cell inhibition with the increase of DOX concentration. Compared with free DOX, the cell viability decreased as the DOX concentration increased for both DOX-L and HA-DOX-L. Moreover, when the DOX concentration reached ≥ 5 μg/mL, both DOX-L and HA-DOX-L experienced similar cytotoxicity to free DOX. These results should be due to the prolonged release of DOX from DOX-L and HA-DOX-L.

According to the results of Fig.5, the growth inhibition concentration (IC_{50}) of free DOX, DOX-L and HA-DOX-L were calculated. The IC_{50} of free DOX solution to HCT-116 cells was 0.15 ± 0.01 μg/mL, which was lower than that of HA-DOX-L (0.19 ± 0.01 μg/mL) and DOX-L (0.46 ± 0.02 μg/mL). The lowest IC_{50} value of free DOX might be a result of passive diffusion with high concentration gradient under *in vitro* conditions, while the highest IC_{50} values of DOX-L may be derived from their internalization through an endocytosis pathway(Hayashi et al. 2013). Moreover, liposomes might have undergone a sustained-release process, which result in a slow diffusion and release of DOX from liposome into external environment(Zong et al. 2014). In addition, from IC_{50} values, HA-DOX-L had a more pronounced toxicity than DOX-L, and slightly lower toxicity than free DOX. This could be

explained that HA-DOX-L was specifically recognized by the CD44 on the surface of HCT-116 cells and the drug was taken up by the cells *via* active transport. Thus, HA-DOX-L had a potential active targeting to CD44 overexpressed tumor cells.

Intracellular distribution of DOX -L and HA-DOX-L

The intracellular distribution and uptake of DOX-L and HA-DOX-L by HCT-116 cells were confirmed *via* confocal microscopy. Since DOX itself is fluorescent, it was used directly to measure cellular uptake without additional markers. Therefore, the fluorescence intensity was proportional to the amount of DOX internalized by the cells. Fig.6 showed the cellular fluorescence image of HCT-116 cells after being incubated with HA-DOX-L (Fig.6a) and DOX-L (Fig.6b) for 6 h. It was obvious that the red fluorescence intensity in cells exposed to HA-DOX-L was much stronger than DOX-L. Besides, the fluorescence was much clearly observed in the entire intracellular matrix (Fig.6a) when HCT-116 cells were exposed to HA-DOX-L. This could be explained by the higher affinity of HA coated liposome to cells. HA was specifically recognized by CD44 on the surface of HCT-116 cells, which enhanced the intracellular uptake of HA-DOX-L(Negi et al. 2015). This result was in agreement with the higher cytotoxicity of the HA-DOX-L compared to DOX-L observed by MTT analyses. And the uptake of liposome into HCT-116 cells was related to its anti-tumor efficacy.

Conclusions

The over expression of the HA receptor, CD44, on a variety of tumors makes HA a potentially interesting ligand for targeted therapy of such tumours. In this study, HA decorated DOX loaded liposomes were prepared by pH-transmembrane method, where the optimal ratio of DOX/lipids was 1:5. The average size of this system was less than 120 nm with uniform distribution and high EE (> 97%). Although HA-DOX-L has prolonged the circulation time of the drug, it was still more efficacious in habiting the CD44 over expressed cancer cells than non-HA coated DOX-L, which was confirmed by MTT and intracellular distribution assays. Therefore, HA-DOX-L could function as tumor-targeted carrier for DOX. Further studies are in progress to deeply investigate the HA-DOX-L intracellular trafficking and to evaluate their *in vivo* antitumoral activity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Anabousi S, Laue M, Lehr CM, Bakowsky U, Ehrhardt C(2005). Assessing transferrin modification of liposomes by atomic force microscopy and transmission electron microscopy. Eur. J. Pharm. Biopharm. 60:295-303. https://doi.org/10.1016/j.ejpb.2004.12.009.

Andresen TL, Jensen SS, Gensen KJ(2005). Advanced strategies in liposomal cancer therapy: Problems and prospects of active and tumor specific drug release. Progress in Lipid Research. 44:68-97. https://doi.org/10.1016/j.plipres.2004.12.001.

Bourguignon LYW, Singleton PA, Zhu HB, Zhou B(2002). Hyaluronan promotes signaling interaction between CD44 and the transforming growth factor beta receptor I in metastatic breast tumor cells. J. Biol. Chem. 277:39703-39712. https://doi.org/10.1074/jbc.M204320200.

Cullis PR, Bally MB, Madden TD, Mayer LD, Hope MJ(1991). pH gradients and membrane transport in liposomal systems. Trends Biotechnol. 9:268-272. https://doi.org/10.1016/0167-7799(91)90088-Y.

Csoka AB, Frost GI, Stern R(2001). The six hyaluronidase-like genes in the human and mouse genomes. Matrix Biol. 20:499-508. https://doi.org/10.1016/S0945-053X(01)00172-X.

Dong J, Jiang DH, Wang Z, Wu GZ, Miao LY, Huang LX(2013). Intra-articular delivery of liposomal celecoxib–hyaluronate combination for the treatment of osteoarthritis in rabbit model. International Journal of Pharmaceutics. 441:285-290. https://doi.org/10.1016/j.ijpharm.2012.11.031.

Fan YC, Du WW, He B, Fu FY, Yuan L, Wu HN, Dai WB, Zhang H, Wang XQ, Wang JC, Zhang X, Zhang Q(2013). The reduction of tumor interstitial fluid pressure by liposomal imatinib and its effect on combination therapy with liposomal doxorubicin. Biomaterial. 34:2277-2288. https://doi.org/10.1016/j.biomaterials.2012.12.012.

Hayashi K, Tatsui T, Shimanouchi T, Umakoshi H(2013). Enhanced Cytotoxicity for Colon 26 Cells Using Doxorubicin-Loaded Sorbitan Monooleate (Span 80) Vesicles. Int. J. Biol. Sci. 9:142-148. https://doi.org/10.7150/ijbs.5453.

Kim E, Yang J, Park J, Kim S, Kim NH, Yook JI, Suh JS, Haam S, Huh YM(2012). Consecutive targetable smart nanoprobe for molecular recognition of cytoplasmic microRNA in metastatic breast cancer. ACS Nano. 6:8525-8535. https://doi.org/10.1021/nn300289u.

Li F, Yang R, Weng Y, Tang X(2009). Preparation and evaluation of lyophilized liposome-encapsulated bufadienolides Drug development and industrial pharmacy. 35:1048-1058. https://doi.org/10.1080/03639040902762987.

Montero MT, MartÍ A, Hernàndez-Borrell J(1993). The active trapping of doxorubicin in liposomes by pH gradient: photon correlation spectroscopy and fluorimetric study. Int. J. Pharm. 96:157-165. https://doi.org/10.1016/j.ijpharm.2012.04.011.

Nakamura K, Yoshino K, Yamashita K, Kasukawa H(2012). Designing a novel in vitro drug-release-testing method for liposomes prepared by pH-gradient method. Int. J. Pharm. 430:381-387. https://doi.org/10.1016/j.ijpharm.2012.04.011.

Negi LM, Jaggi M, Joshi V, Ronodip K, Talegaonkar S(2015). Hyaluronan coated liposomes as the intravenous platform for delivery of imatinib mesylate in MDR colon cancer. Int. J. Biol. Macromol. 73: 222-235. https://doi.org/10.1016/j.ijbiomac.2014.11.026.

Nielsen SS, Toft KN, Snakenborg D, Jeppesen MG, Jacobsen JK, Vestergaard B, Kutter JP, Arleth L(2009). BioXTAS RAW, a software program for high-throughput automated small-angle X-ray scattering data reduction and preliminary analysis. J Appl Crystallogr. 42:959-964. https://doi.org/10.1107/S0021889809023863.

Paliwal SR, Paliwal R, Agrawal GP, Vyas SP(2011) Liposomal nanomedicine for breast cancer therapy. Nanomedicine. 6:1085-1100. https://doi.org/10.2217/nnm.11.72.

Park JH, Cho HJ, Yoon HY, Yoon IS, Ko SH, Shim JS, Cho JH, Park JH, Kimh K, Kwon IC, Kima DD(2014). Hyaluronic acid derivative-coated nanohybrid liposomes for cancer imaging and drug delivery. Journal of Controlled Release. 174: 98-108. https://doi.org/10.1016/j.jconrel.2013.11.016.

Peer D, Margalit R(2004) Tumor-targeted hyaluronan nanoliposomes increase the antitumor activity of liposomal Doxorubicin in syngeneic and human xenograft mouse tumor models. Neoplasia. 6:343-353. https://dx.doi.org/10.1593/neo.03460.

Rahman YE, Cerny EA, Tollaksen SL, Wright BJ, Nance SL, Thomson JF(1974) Liposome-encapsulated actinomycin D: potential in cancer chemotherapy. Proe soc exp boil med.146:1173-1176.

Silvia A, Carlotta L, Elisa DP, Chiara C, Nicolas T, Barbara S, Massimo D, Ilaria D, Elias F, Luigi C, Marta P(2013). Hyaluronic acid-coated liposomes for active targeting of gemcitabine. European Journal of Pharmaceutics and Biopharmaceutics. 85:373-380. https://doi.org/10.1016/j.ejpb.2013.06.003.

Toole BP(2004) Hyaluronan: from extracellular glue to pericellular cue. Nat. Rev. Cancer. 4:528-539. https://www.nature.com/articles/nrc1391.

Wu W, Zhang QJ, Wang JT, Chen M, Li S, Lin ZF, Li JS(2014). Tumor-targeted aggregation of pH-sensitive nanocarriers for enhanced retention and rapid intracellular drug release. Polym. Chem. 5:5668-5679. https://doi.org/10.1039/C4PY00575A.

Yang XY, Li YX, Li M, Zhang L, Feng LX, Zhang N(2013). Hyaluronic acid-coated nanostructured lipid carriers for targeting paclitaxel to cancer Cancer Lett. 334:338-345. https://doi.org/10.1016/j.canlet.2012.07.002.

Zöller M(2011) CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? Nat. Rev. Cancer. 11:254-267. https://doi.org/10.1038/nrc3023.

Zhang J, Chen XG, Li YY, Liu CS(2007) . Self-assembled nanoparticles based on hydrophobically modified chitosan as carriers for doxorubicin. Nanomedicine. 3: 258-265. https://doi.org/10.1016/j.nano.2007.08.002.

Zhang WL, Wang GJ, Falconer JR, Baguley BC, Shaw JP, Liu JP, Xu HT, See E, Sun JG, Aa J, Wu ZM(2014). Strategies to Maximize Liposomal Drug Loading for a Poorly Water-soluble Anticancer Drug. Pharm. Res. 32:1451-1461. https://doi.org/10.1007/s11095-014-1551-8.

Zong T, Ling M, Gao H, Wei C, Zhu P, Shi K, Chen J, Yang W, Gao F, Qin H(2014). Synergistic dual-ligand doxorubicin liposomes improve targeting and therapeutic efficacy of brain glioma in animals. Mol. Pharmaceutics. 11:2346-2357. https://doi.org/10.1021/mp500057n.

Figure Captions

Fig. 1 Characterizations of DOX-L and HA-DOX-L by DLS and TEM measurements: DOX-L (a, b) ; HA-DOX-L (c, d) and e is an enlarge image of HA-DOX-L

Fig. 2 SAXS curves of blank liposome, DOX-L and HA-DOX-L

Fig. 3 Physical characterizations of DOX-L and HA-DOX-L with time dependence in 6 months: size and PDI changes (a); EE and DL changes (b). Data are presented as the mean ± standard deviation (n = 3)

Fig. 4 *In vitro* release profile of DOX from DOX-L and HA-DOX-L in PBS (pH = 7.4) at 37 ± 0.5 °C. Data are presented as the mean ± standard deviation (n = 3)

Fig. 5 Cytotoxicity on HCT-116 cells as determined by MTT assay (48 h incubation). Data are presented as the mean ± standard deviation (n = 3)

Fig. 6 Confocal microscopy images of HCT-116 cells incubated with HA-DOX-L (a) and DOX-L (b) at 37 °C for 6 h