Preparation and Efficacy of Tumor Vasculature-Targeted Doxorubicin Cationic Liposomes Coated by *N*-Trimethyl Chitosan

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Received 17 June 2010; accepted 2 November 2010
DOI 10.1002/app.33944
Published online 16 March 2011 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: Cationic liposomes (CLs) can accumulate in tumor vascular endothelial cells (VECs) to show high selective targeting ability. Therefore, chemotherapeutic agent-loaded CLs are considered as new therapeutic vehicles to enhance the treatment efficacy. This study investigated the effect of *N*-trimethyl chitosan (TMC), one of derivatives of chitosan with positive charge determined by its degree of quaternization (DQ), on preparing doxorubicin (DOX)-loaded CLs. TMCs with various DQ, i.e., 20% (TMC20), 40% (TMC40), and 60% (TMC60) were synthesized and characterized by ^{1}HNMR. DOX-loaded liposomes (DOXL) were prepared by ammonium sulfate gradients followed by TMC-coating to obtain TMC-coated DOXL with various positive surface charges. The morphology, size, ζ-potential and drug release *in vitro* of TMC-coated DOXL were studied compared with those of DOXL. Human umbilical vein endothelial cells (HUVECs) as cell model, the vascular targeting ability of TMC-coated DOXL was evaluated *in vitro*. A solid tumor, formed by implantationmurine hepatoma cells (H_{22}) into mice, as tumor model, the tumor inhibition rate and tumor histological sections stained by HE of TMC-coated DOXL group were researched compared with those of free DOX and DOXL group. It was found that with the increase of TMC's DQ, the positive surface charge of TMC-coated DOXL was enhanced accordingly, which had little effect on DOX release *in vitro* while led to the significant increase of DOX uptake by HUVECs *in vitro* and the treatment effect on solid tumor *in vivo*. Especially, TMC-coated DOXL showed better targeting ability to the nuclei compared with free DOX and DOXL, which could further enhance the efficacy of DOX *in vivo*. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 2149–2156, 2011

Key words: *N*-trimethyl chitosan; cationic liposomes; tumor vascular targeting; doxorubicin

INTRODUCTION

It has been proven that the tumor vasculature is relatively more dynamic and permeable than healthy host tissue. Meanwhile, vascular endothelial cells (VECs) play an important role in the neovascularization.[1] In tumor vasculature, there is above 25% VECs in the state of caryocinesis leading to the quick formation of new vessels, which supply oxygen and nutrients for the tumor cell growth. Therefore, vascular targeting is proposed as a new therapeutic concept based on the destruction of tumor microvasculature. The mechanisms are mainly on account of high tumor vasculature targeting vehicles combined with destructive effect of chemotherapeutic agents on VECs, which lead to decrease of the nutrition supply and final necrosis of tumor cells.[2] Among so many vehicles for antitumor drugs, cationic liposomes (CLs) have been reported to enable target dynamic tumor vasculature leading to the selective delivery of antiangiogenic agents.[3–6] Fluorescence microscopy has verified that CLs could target intratumor capillary vessels instead of neoplastic cells and enhance tumor inhibition rate significantly when compared with neutral and anionic liposomes.[7] The electrostatic interaction between positive surface charge of CLs and negative charge components in tumor VECs, such as phospholipids, proteoglycans and membrane protein, etc, may be a key factor in the tumor vascular targeting ability.[8–10]

However, one basic problem with CLs is toxicity. This is normally closely associated with the charge ratio of the cationic lipid species in the formulation and the charge of the anionic nucleic acid-based drug. Higher charge ratios are generally more toxic to a variety of cell types, including cancer cell lines.[11] In those related studies that have been reported, fluorescently labeled 1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP) and dimethyl dioctadecylammonium bromide (DDAB) are the only cationic lipids usually used to prepare CLs. It is very convenient to observe the tumor vasculature targeting of the drug-loaded liposomes because of their fluorescence. However, with the amount increase of the positive charge materials in the

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formulation of CLs, the cytotoxicity is increased significantly.[2,12] Therefore, it is very necessary to look for better biocompatible positive charge components for CLs with promising tumor vascular targeting ability.

Chitosan (CS) is the only polycationic polysaccharide in nature which has been attracting much interest in pharmaceutical application. Because of its poor solubility (only soluble in pH $<$ 6.5 solution), some derivatives of CS with wider soluble pH range have been synthesized successfully. Among them, *N*-trimethyl chitosan (TMC) is the most frequently studied and used because of its well-defined structure, improved solubility and easy preparation.[13] Moreover, the biocompatibility of TMC is better than that of CS.[14] The positive charge of TMC as determined by its degree of quaternization (DQ) is an important factor determining its potential application in preparation of microparticles with positive surface charge.

The aim of our study was to take advantage of TMC's positive charge and promising biocompatibility to prepare CLs. TMC with DQ of 20, 40, and 60%, respectively, i.e., TMC20, TMC40, and TMC60, were synthesized and characterized by ^{1}HNMR. Doxorubicin (DOX) as model drug, liposomes were prepared followed by TMC-coating. The properties *in vitro* of the CLs were studied. The vascular targeting ability was evaluated *in vitro* and the antitumor effect of the CLs was also investigated *in vivo*.

MATERIALS AND METHODS

General chemicals and polymers

CS (Mw210 kDa, DD $>$ 95%) was bought from Haipu Biotechnology Co. Ltd. (Qingdao, China). DOX was purchased from Huafenglianbo Technology Co. Ltd. (Beijing, China). Lecithin and cholesterol were both obtained from Hufeng Biotechnology Co. Ltd. (Shanghai, China). All the other chemicals were of analytical grade.

Mice

Healthy male Kunming species mice weighing 20 $\pm$ 2g were supplied by the Experimental Animal Breading Center of Medical College of Wuhan University. All of the procedures were performed according to the NIH guideline.

Cell lines

Murine hepatoma cells (H_{22}) were bought from the Conservation Centre of Wuhan University. Human umbilical vein endothelial cells (HUVECs) were gifted from Wenzhou Medical College.

Synthesis and characterization of TMCs

TMCs with DQ of 20% (TMC20), 40% (TMC40), and 60% (TMC60) were synthesized by varying the number of times and durations of reaction steps as reported by D.Snyman,[15] and characterized by ^{1}HNMR in D_2O at 80°C using a 600-MHz spectrometer (Varian unity Inova, USA). Their DQs were calculated with the following equation[16] $DQ(\%)=[(\int TM/\int H)\times(1/9)]\times 100$, where $\int$TM is the integral of the trimethyl amino group peak at 3.7 ppm and $\int$H is the integral of the ^{1}H peaks from 5.0 to 6.0ppm.

Preparation and analysis of DOX-loaded liposomes coated by TMCs

DOX-loaded liposomes (DOXL) were prepared by transmembrane ammonium sulfate gradients method[17] followed by drop-wised into the 0.2% (w/w) TMC20, TMC40, and TMC60 water-solution, respectively, at the rate of 1 mL/min under magnetic agitation of 30 rpm. The volume ratio of TMC solution and DOXL suspension was 4:1.[18] Agitation was kept for another 10 min to obtain TMC-coated DOXL. The DOXL and TMC-coated DOXL suspensions were both stored at 4°C under argon.

The morphology of the liposomes was observed using a transmission electron microscope (TEM-100X II, Electron Co., Japan). The particle size and ζ-potential of the liposomes were analyzed by a Malven Zetasizer (Zetasizer 3000HS, Malven Instruments, Germany). G-50 sephadex column (1.6 cm $\times$ 20 cm) was used to separate free DOX from the liposomes. The entrapment rate of the liposomes was determined by a high-performance liquid chromatography (HPLC) using an UV-visible detector (254 nm for DOX, Agilent1100, USA). DOX release rate from the liposomes *in vitro* was determined by dialyzing method.

Vascular endothelial cell binding of the liposomes

HUVECs were chosen as cell samples *in vitro* in the study.[1] Diamino-phenyl- indole (DAPI) for nucleic staining, laser confocal microscope (Leica TCS SP2, Germany) was applied to investigate DOX uptake by HUVECs and DOX transmission from the cytoplasm to the nuclei.

HUVECs with the concentration of 2 $\times$ 10^4 cells/mL were put on sterile cover slips and then put into 24-well plates. The cells were cultivated in a CO_2 incubator (Thermo Forma, USA) at 37. in a 50% CO_2/95% humidified air atmosphere in RPMI1640 culture medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 IU/mL penicillin and 100 mg/mL streptomycin. After drawing-off the culture medium, 200 μL fresh culture medium without FBS was

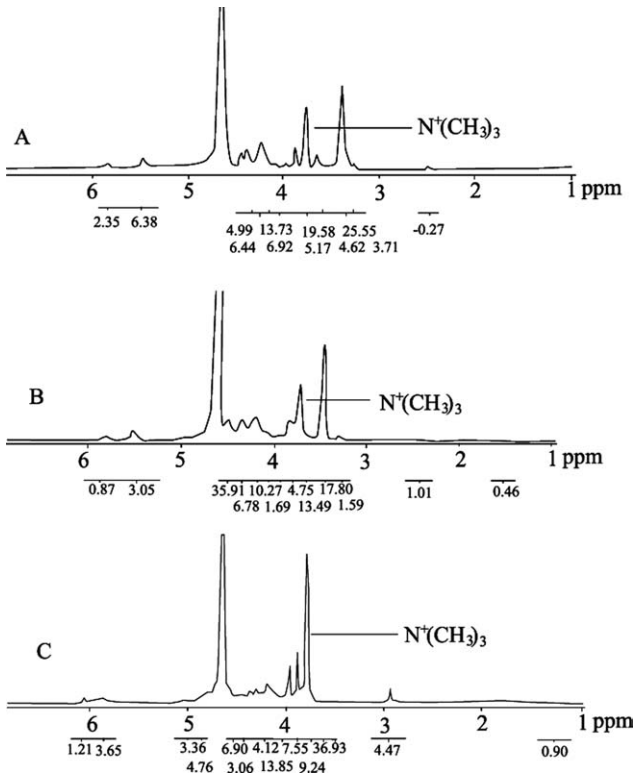

Figure 1 ^{1}H NMR spectra of synthesized TMCs (A: TMC20, B: TMC40 and C: TMC60).

added followed by the addition of TMC-coated DOXL, DOXL or free DOX (20 μL each, the final concentrations of DOX were all adjusted to 10 μg/mL). The cells were then incubated at the same condition as described above. At the predetermined time intervals of 0.5 h, 1 h, 2 h, and 4 h, the culture medium was removed. The cells were next washed with PBS (pH7.4) to remove unwanted cellular debris and unbound DOX or liposomes, and fixed with 4% paraform for 15 min. After the rupture of membrane with 0.1% TritonX-100 PBS, 20 μg/mL DAPI (340/488 nm) methanol solution was added to stain the nuclei at 37°C for 15 min. After the excess DAPI solution was washed out with methanol, the slips were mounted onto a glass microslide with 50% glycerin and DOX transmission from the cytoplasm to nuclei was observed under the laser confocal microscope with excitation wavelength of 488 nm and emission at 543 nm. The nuclei were located by DAPI which was excited with 340 nm wavelength. The red fluorescent gap and the blue fluorescent gap were overlapped to determine DOX uptake by HUVECs at the different time intervals.

Antitumor activity in tumor-bearing mice

The mice were housed in single cages and had free access to tap water and standard laboratory food throughout the experiments.

The anabiotic H_{22} cells were seeded in abdominal cavity of the healthy mice (0.4 mL each). The abdominal dropsy was drawn-off and diluted with physiological saline (PS) followed by seeded in abdominal cavity of the other healthy mice. Repeat three times. Finally, the ivory abdominal dropsy was drawn-off and then adjusted to 1×10^6 cells/mL with PS to obtain neoplastic cell suspension.

Sixty healthy mice were randomly divided into six groups with ten in each. Every mouse was injected 0.2 mL the neoplastic cell suspension into the right limb armpit. The whole injection process was finished in one hour. After five days period, PS, DOX, DOXL, or TMC-coated DOXL was injected via tail vein with the amount of injected DOX of 5 mg/kg. Then after 12 days, the mice were sacrificed by cervical vertebra dislocation. The tumors were stripped, weighed, and mounted with 10% neutrol formalin. The average tumor weights (W) were calculated and the tumor inhibition rate was evaluated by the following equation: tumor inhibition rate $= (W_{\text{control group}} - W_{\text{treatment group}})/W_{\text{control group}} \times 100\%$, where control group was injected with the same volume of PS, and treatment group was injected with DOX, DOXL, or TMC-coated DOXL, respectively. The tumor histological sections were stained by HE and the pathological changes were observed under a light microscope.

Statistical analysis

Data were expressed as the mean value ± S.D. To determine the significant difference between different experimental groups nonparametric Mann-Whitney U-test was used. Statistical significance was established at P value ≤ 0.05. Analysis was performed using the statistical package SPSS 12.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Characterization of synthesized TMCs

In Figure 1, typical ^{1}HNMR spectra of TCM20, TMC40, and TMC60 were depicted. The DQ of TMC20, TMC40, and TMC60 was 25%, 38%, and 59%, respectively, suggesting the successful

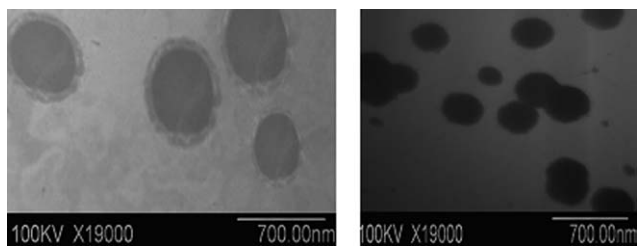

Figure 2 TEM images of liposomes prepared (A) with TMC-coating and (B) without TMC-coating (100 KV ×19,000, 700 nm).

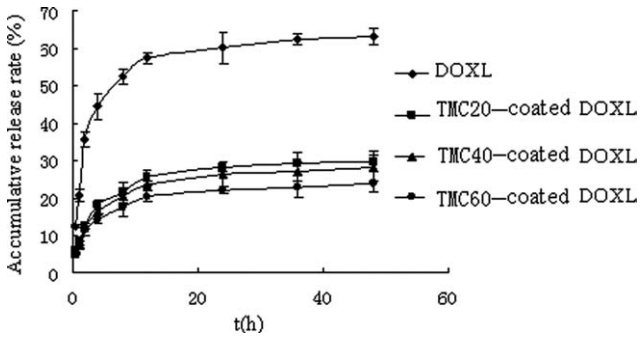

Figure 3 The average curves of accumulated release of DOX from DOXL and TMC-coated DOXL *in vitro.*

synthesis of TMCs with various DQ. A 0.3-ppm shift towards low chemical shift was observed in our detection as compared with those standard spectra. Therefore, in the DQ calculating equation, $\int H$ is the integral of the ^1H peaks from 5.0 to 6.0 ppm and $\int TM$ is the $N^+(CH_3)_3$ peak at 3.7 ppm.[13]

Properties of the DOX-loaded liposomes *in vitro*

Gradients of ammonium sulfate in liposomes could be used to obtain active and high loading of DOX into the aqueous compartment of liposomes with prolonged storage periods.[17] Therefore, DOXL was prepared according to the technology in the study.

TABLE I
Mean Diameter, Zeta-Potentials, and Entrapment Rate of Various DOX Liposomes

Liposomes	Mean size (nm)	Zeta potential (mv)	Entrapment rate (%)
DOXL	164.1 ± 0.1	−9.6 ± 0.5	62.3%
TMC20-coated DOXL	172.1 ± 0.1	6.9 ± 0.2*	65.2%
TMC40-coated DOXL	183.9 ± 0.2	9.7 ± 0.7*	60.7%
TMC60-coated DOXL	185.5 ± 0.2	16.1 ± 0.6*	66.3%

* Note: compared with DOXL, $P < 0.05$.

Typical examples of TEM images of the liposomes with and without TMC-coating (A and B, respectively) are shown in Figure 2, clearly exhibiting the well-defined TMC-coating layer. The mean size, ζ-potential and entrapment rate of DOXL and TMC-coated DOXL are listed in Table I. Before TMC-coating, the Zeta potential of DOXL was negative [(−9.6 ± 0.5)mv], and changed into positive after TMC-coating, suggesting the successful preparation of cationic liposomes. Furthermore, with the increase of

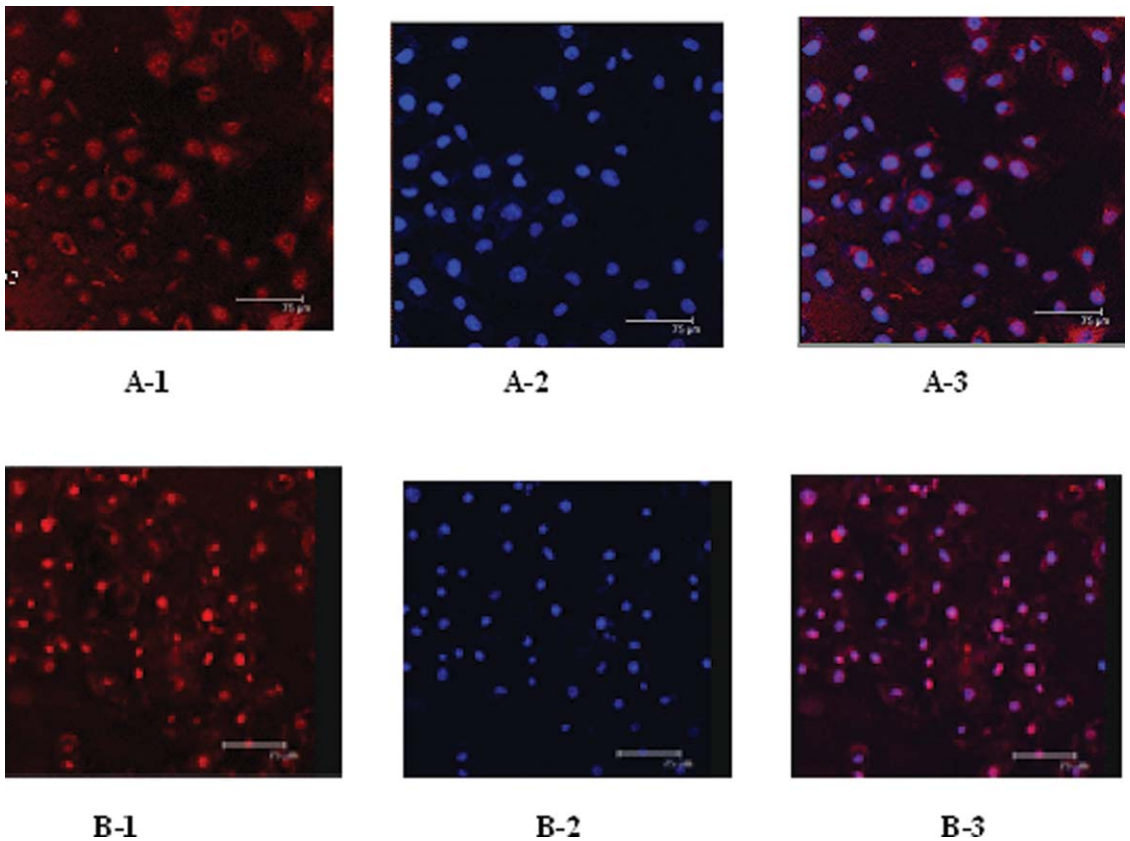

Figure 4 The uptake of DOXL by HUVECs with (B) or without (A) TMC60: A-1 and B-1were the photos of the red fluorescence of DOX; A-2 and B-2 were the photos of DAPI for nuclei staining with blue fluorescence, A-3 and B-3 were the overlapped photos of the above two. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

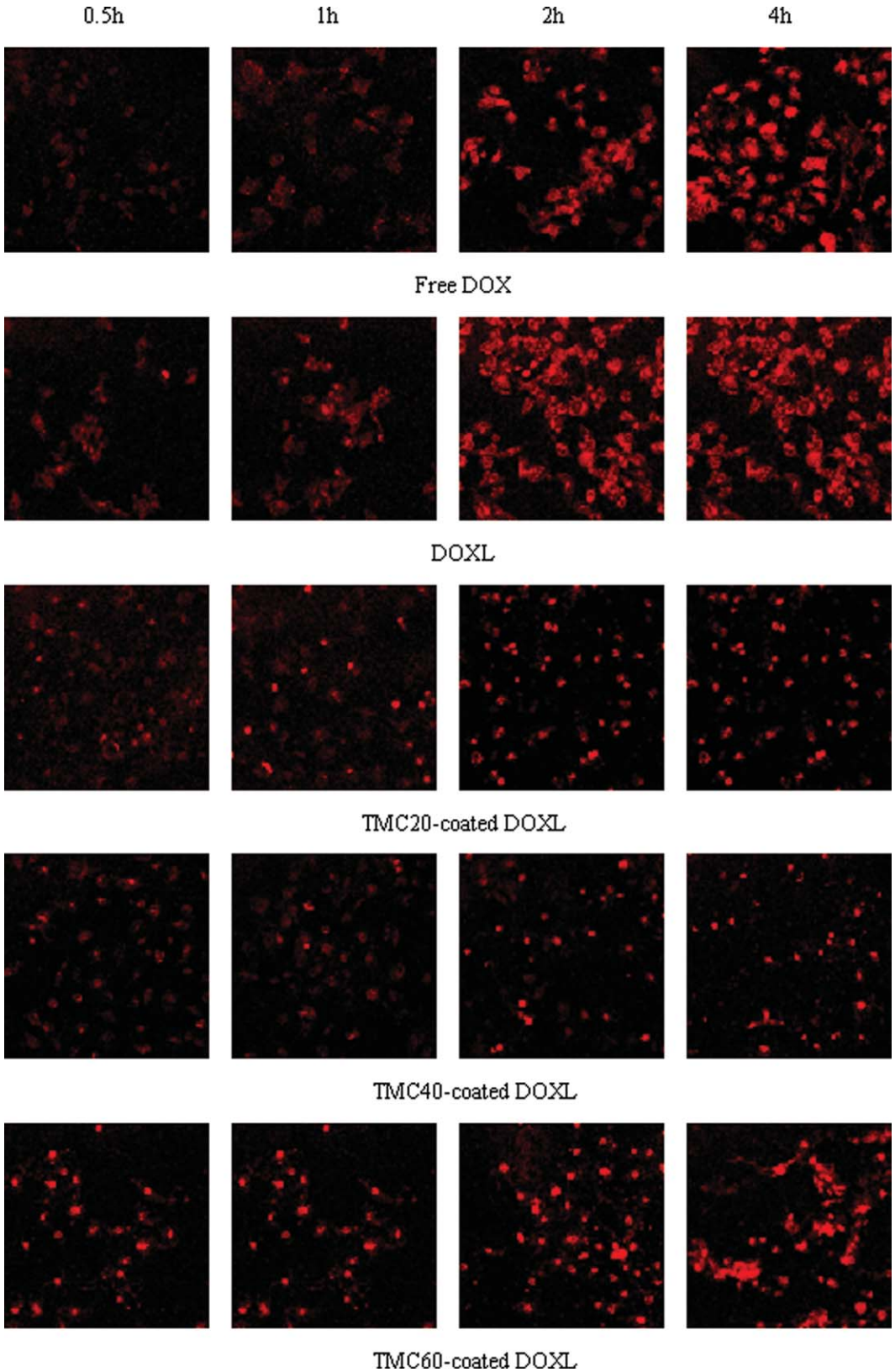

Figure 5 The fluorescence images of DOX uptake by HUVECs after incubation with free DOX, DOXL, and TMC-coated DOXL for different time periods. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

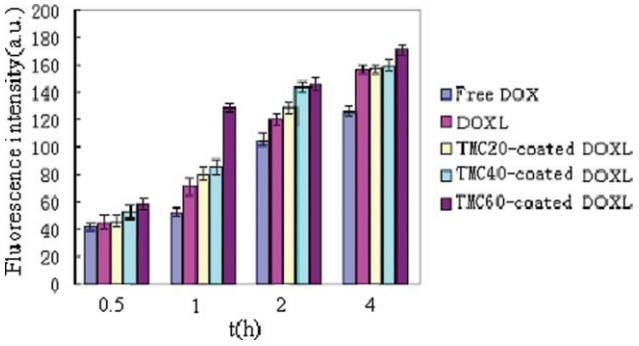

Figure 6 The fluorescence intensity of HUVECs after incubation with free DOX, DOXL, and TMC-coated DOXL for different time periods. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the DQ of TMC, the absolute value of ζ-potential was increased accordingly. It is therefore reasonable speculate that liposomes with higher negative surface charge could interact more easily with TMCs through electrostatic attraction to form more stable coating layer. After TMC-coating, the mean size of the liposomes was increased slightly because of the TMC-coating layer; however, the entrapment rate of the liposomes had little change.

The average accumulated release profiles of DOX from the different liposomes *in vitro* were presented in Figure 3, showing the significant decrease of DOX release rate and amount from TMC-coated liposomes compared with from DOXL, because of the further sustain releasing effect of the TMC coating layer.[19] After curve fitting, it was found that DOX release from the liposomes was all accorded with Higuchi equation, i.e., $Q = 21.854 + 7.2745t^{1/2}$, $r = 0.9184$ for DOXL, $Q = 7.6315 + 3.7863t^{1/2}$, $r = 0.9292$ for TMC20-coated DOXL, $Q = 6.9647 + 3.5709t^{1/2}$, $r = 0.9318$ for TMC40-coated DOXL and $Q = 7.3451 + 2.7665t^{1/2}$, $r = 0.9357$ for TMC-60-coated DOXL, where the stable release rate was 7.2745, 3.7863, 3.5709, and 2.7665 μg/h, respectively. It was proved that TMC-coating could further sustain DOX release from the liposomes *in vitro*; however, the DQ of TMC had little effect on the release rate.

VECs association of the liposomes

At the excitation wavelength of 340 nm, DOX showed red fluorescence while DAPI was with blue fluorescence to locate the nuclei. Under the laser confocal microscope, the red fluorescence gap and the blue one were overlapped to show DOX uptake by HUVECs. Figure 4 shows the uptake state after 4-h incubation of DOXL and HUVECs with or without the addition of TMC60.[20] The red fluorescence of DOX was exhibited in A-1 and B-1, the blue fluorescence of nuclei with DAPI was shown in A-2 and B-2, and the overlapped image of A-1 and A-2, or B-1 and B-2 was displayed in A-3 and B-3, respectively. Figure 4(A-3) displayed clearly that the red fluorescence mainly distributed around the blue one, showing that DOX was mainly located in the cytoplasm with little entered the nuclei. However, in Figure 4(B-3), the blue fluorescence was almost displaced by the red one, suggesting that DOX could enter the nuclei easily. Those results proved that DOX mainly distributed in the cytoplasm with the help of common lipsomes while could further enter the nuclei with the addition of TMC, showing improved cell targeting ability.

At various incubation times, the DOX uptake by HUVECs of free DOX, DOXL and TMC-coated DOXL was exhibited in Figure 5, and the fluorescence intensity was shown in Figure 6. The stronger of the red fluorescence intensity, the more DOX was bound with HUVECs.[21] Figure 6 exhibited that at all observing times, the fluorescence intensity of free DOX and DOXL group was both weaker than those of TMC-coated DOXL groups, and with the increase of TMC's DQ, the uptake was enhanced accordingly. Especially at 1h of uptake, the difference was the most significant. More interestingly, the fluorescence images of TMC-coated DOXL were punctiform while those of free DOX and DOXL were massive, which showed the DOX distribution in nuclei with TMC-coating while just in cytoplasm without TMC-coating. Therefore, the DOX-loaded cationic liposomes with TMC-coating could enhance the nucleic uptake significantly. The results also proved that the TMC-coated DOXL had promising targeting ability to the VECs *in vitro*, which could enhance the DOX tumor inhibitory efficacy *in vivo*.

Antitumor activity in mice

The tumor inhibition rates of testing groups are listed in Table II. Compared with free DOX and DOXL, TMC-coated DOXL all enhanced the inhibition rate significantly, and the higher DQ of TMC

TABLE II
The Inhibition Rate of Transplanted H_{22}Solid Tumor in Mice Treated by PS, DOX, DOXL, and TMC-Coated DOXL, respectively (n= 10, $\bar{x} \pm s$)

Groups	Body weight of mice (beginning/end, g)	Weight of tumor (g)	Tumor inhibition rate (%)
Physiological saline	20.5/29.4	1.36 ± 0.42	–
DOX	21.2/27.8	0.78 ± 0.49	42.7
DOXL	20.8/28.9	0.87 ± 0.29	36.4
TMC20-coated DOXL	21.4/30.5	0.62 ± 0.25	54.8*
TMC40-coated DOXL	20.2/27.6	0.59 ± 0.28	57.0*
TMC60-coated DOXL	20.7/25.2	0.49 ± 0.18	64.3*

* Note: compared with free DOX and DOX L ($P < 0.05$).

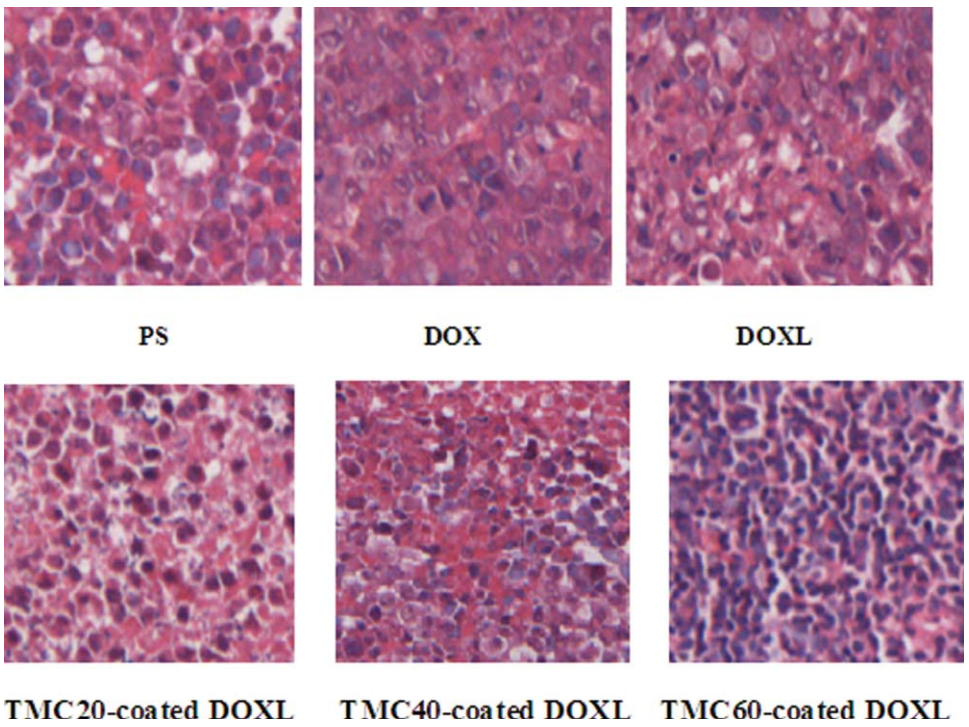

Figure 7 Tissue section of H_{22} hepatocarcinoma in mice (HE dyeing, ×200). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

was, the better the inhibition rate was. The results showed the direct proportion relationship between the tumor targeting ability and the positive surface charge of the cationic liposomes.[20]

The HE-stained histological section images of H_{22} transplantation tumor of mice hepatoma are displayed in Figure 7. Among them, PS control group showed the following states: the most active tumor cell growth, lightest hemorrhage and necrosis, biggest tumor nuclei, most caryokinesis and vascular proliferation. Compared with PS group, DOX and DOXL group both showed fewer tumor cells and blood vessels accompanied with slight focus necrosis. Compared with the above groups, TMC-coated DOXL group all displayed the severest tumor cell necrosis with part hemorrhage. Especially, great pieces of necrosis with infiltration by many homeocytes and macrophages could be found in TMC60-coated DOXL group, and in tumor interstitium, there was fewest blood vessels could be found. Meanwhile, in PS group, tumor tissue infiltration was the deepest into the fat and muscle, even invading the thoracic cavity. In TMC-coated DOXL groups, the volume of tumor was the smallest and the infiltration was the lightest only into the fat accompanied with part liquate necrosis. The results further proved that TMC-coated DOXL had strong antitumor efficacy, which could decrease the tumor vascular number and volume, and promote the tumor necrosis.

CONCLUSIONS

In this study, DOX-loaded cationic liposomes were prepared by TMC-coating. The positive surface charge of the liposomes was raised with the increase of TMC's DQ. DOX release from the TMC-coated DOXL was slower than that from DOXL *in vitro*, which was independent on TMC's DQ. Compared with free DOX and DOXL, TMC-coated DOXL showed significant effect on the DOX uptake by HUVECs *in vitro* and the tumor inhibition *in vivo*, and the effect was both enhanced with the increase of TMC's DQ. The results verified that cationic liposomes could be obtained easily by cationic polymer coating with promising tumor vascular targeting ability and possible better safety than those prepared from total synthesized cationic materials. Studies on the bio-safety and biocompatibility of the TMC-coated cationic liposomes are undergoing in our lab.

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