JOURNAL OF Applied Polymer SCIENCE

Cytotoxicity of Cationic Liposomes Coated by *N*-Trimethyl Chitosan and Their *In Vivo* Tumor Angiogenesis Targeting Containing Doxorubicin

Xian-Xi Guo,[1] Wen He,[1] Xiao-Jie Zhang,[2] Xian-Ming Hu[2]

[1]Department of Pharmacy, Renmin Hospital of Wuhan University, Wuhan 430060, People's Republic of China

[2]School of Pharmaceutical Science, Wuhan University, Wuhan 430072, People's Republic of China

Correspondence to: X.-M. Hu (E-mail: xmhu@whu.edu.cn)

ABSTRACT: Doxorubicin (DOX)-loaded cationic liposomes (DOXL) coated by *N*-trimethyl chitosan (TMCs) has been previously shown to enhance DOX uptake by human umbilical vein endothelial cells (HUVECs) *in vitro* and the tumor inhibition on solid tumor *in vivo*, and the effects were both enhanced with the degree of quaternization (DQ) increase of TMCs. The aim of the present work is to study the cytotoxicity of the blank cationic liposomes (CLs) coated by TMCs with various DQ on L-929 mouse fibroblasts, by MTT assay, using the relative proliferation rate as the indicator, and the toxicity extent was classified according to the evaluation criteria of United States Pharmacopoeia. Furthermore, the *in vivo* tumor angiogenesis targeting of DOXL coated by TMC60 was studied. It was found that with the increase of TMCs concentration and DQ, cytotoxicity was increased accordingly. However, the cell proliferation rates of TMCs-coated CLs with TMCs concentrations of 0.02% and 0.05%(w/w) were all above 80%, even the concentration of TMC20 was increased to 0.2%(w/w), the cell proliferation rate was still above 80%, showing noncytotoxicity. The mouse H_{22} tumor model was established by transplanted tumor experiment. *In vivo* fluorescence in tumor tissue was investigated through the tail vein injection of fluorescein isothiocyanate conjugated dextran at the 7th day after the administration of different DOX preparations. Compared with DOX solution and uncoated DOXL, the mice given TMC60-coated DOXL showed tumor angiogenesis with good shape, uniform arrangement, and small vascular branches, and the vascular density was decreased, suggesting promising tumor angiogenesis targeting of TMC60-coated DOXL. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: cytotoxicity; cationic liposomes; *N*-trimethyl chitosan; tumor angiogenesis targeting; doxorubicin

Received 12 August 2011; accepted 13 March 2012; published online
DOI: 10.1002/app.37701

INTRODUCTION

Among various vehicles for antitumor drug delivery, cationic liposomes (CLs) have been reported to accumulate in tumor vascular endothelial cells (VECs) leading to the selective delivery of anti-angiogenic agents.[1–4] The intratumor capillary vessels targeting ability of CLs is much better than that of neutral and anionic liposomes, which results in significant tumor inhibition rate enhancement.[5] Possible mechanisms of the selective targeting ability of CLs include receptor-mediated endocytosis[6] and charge dependent binding and uptake by a potentially altered glycocalyx on endothelial cells of tumor microvessels.[7] Among them, the electrostatic interaction between the positive surface charge of CLs and the negative charge components in tumor VECs, such as phospholipids, proteoglycans, and membrane protein, may be a key factor in the tumor vascular targeting ability of CLs.[8–10] Therefore, chemotherapeutic agent-loaded CLs are considered as promising therapeutic vehicles to enhance the treatment efficacy.

Chitosan (CS) and its positively charged derivatives have been attracting much interest in pharmaceutical application due to their biocompatibility, biodegradability, and low toxicity. Among them, *N*-trimethyl chitosan (TMC) is most frequently studied and applied because of its well-defined structure, improved water-solubility, and easy preparation.[11] It can be used as a promising polycationic polysaccharide to prepare CLs. In our earlier study, doxorubicin (DOX)-loaded CLs (DOXL) coated by TMCs with various degree of quaternization (DQ) were prepared successfully.[12] The positive surface charge of the liposomes was raised with the DQ increase. Compared with free DOX solution and uncoated DOXL, TMCs-coated DOXL exhibited significant enhancement on the DOX uptake by human umbilical vein endothelial cells (HUVECs) *in vitro* and the tumor inhibition *in vivo*, and both effects were enhanced with the increase of DQ. The results confirmed that CLs could be obtained easily by cationic polymer coating with promising tumor vascular targeting ability.

However, the higher charge ratio on CLs are generally more toxic to a variety of cell types, including cancer cell lines.[13,14] The charge ratio is closely related to the concentration of the cationic materials in the formulation. Both CS and TMC have been proved to be nontoxic to several cell types in contrast to 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP).[15] As the biocompatibility of TMC is better than that of CS,[16] it is of great value to evaluate the cytotoxicity of TMCs combined with liposomes as the positive charge coating materials.

The aim of the present study was to evaluate the *in vitro* cytotoxicity on L-929 mouse fibroblasts of the blank CLs coated by TMCs with different DQ and concentration. In addition, the *in vivo* tumor angiogenesis targeting of DOXL coated by TMC60 was investigated to corroborate the tumor vasculature-targeting ability of CLs coated by TMC.

MATERIALS AND METHODS

General Chemicals and Polymers

CS (Mw 210 kDa, DD > 95%) from a shrimp shell was bought from Haipu Biotechnology (Qingdao, China). DOX was purchased from Huafenglianbo Technology (Beijing, China). Lecithin and cholesterol were both obtained from Hufeng Biotechnology (Shanghai, China). RPMI1640 was bought from Baiwo Technology (Shanghai, China). Fetal bovine serum (FBS) was bought from Hangzhou Sijiqing Bioengineering Materials (Hangzhou, China). MTT, DMSO, and FITC-Dextran were purchased from Sigma Company (St. Louis, Mo, USA). Penicillin sodium and streptomycin sulfate for injection were both obtained form Huabei Pharmaceutical (Hebei, China). Trypsinase was purchased from Sino-American Biotec (Beijing, China). Physiological saline (PS) was purchased from Double-crane Pharmaceutical Business (Beijing, China). All other chemicals were of analytical grade and used without further purification.

Mice

Healthy male Kunming species mice weighing 20 ± 2 g were supplied by the Experimental Animal Breading Center of Medical College of Wuhan University. All procedures for animal experimentation were performed according to approved protocols and in accordance with recommendations of the NIH guideline for the proper use and care of laboratory animals.

Cell Lines

Murine hepatoma cells (H_{22}) and L-929 mouse fibroblasts were both bought from the Conservation Centre of Wuhan University.

Synthesis and Characterization of TMCs

TMCs with DQ of 20% (TMC20), 40% (TMC40), and 60% (TMC60) were synthesized by varying the times and durations of the reaction steps as reported by D. Snyman.[17] Briefly, TMCs were synthesized by reductive methylation of CS through a chemical reaction between CS and iodomethane in the presence of sodium hydroxide. The reaction step was repeated several times as appropriate to increase the DQ of the TMCs.

All TMCs were characterized by ^{1}H-NMR. The products were measured in D_2O at 80°, using a 600-MHz spectrometer (Varian unity Inova). The DQ of the synthesized TMCs was calculated using the following formula[18]: $DQ(\%) = [(\int TM/\int H)\times(1/9)]\times 100$, where $\int TM$ is the integral of the trimethyl amino group (quaternary amino) peak at 3.3 ppm and $\int H$ is the integral of the ^{1}H peaks from 5.0 to 6.0 ppm.

Cytotoxicity of the Blank Liposomes Coated by TMCs

Preparation of the TMCs-Coated Liposomes. Liposomes were prepared by film dispersion method followed by TMCs coating. Lecithin, cholesterol, and vitamin E at the ratio of 4 :1 :0.05 (w/w) were dissolved with 30 mL absolute ethanol in a 500 mL pear-shaped flask. Absolute ethanol was then evaporated by a rotary evaporator at 40°C until a thin lipid film was formed. The solvent traces were subsequently eliminated by drying the film at 40°C for another 60 min. The film was further shaking hydrated with 30 mL phosphate buffered solution (PBS, pH 7.4) to obtain liposomal suspension. After several cycles of extrusion by a high-pressure homogenizer (EmusiFlex-C5, Avestin, Canada) using a polycarbonate membrane with the pore size of 200 nm, the suspension was drop wise added at the rate of 1mL/min into 0.2% (w/w) TMC20, TMC40, and TMC60 water solution, respectively under magnetic agitation of 30 rpm to obtain the TMCs-coated CLs. For the TMC60-coated CLs, TMC60 concentration of 0.02, 0.05, 0.07, 0.10, and 0.15% (w/w) were also used to generate CLs with varying concentrations of TMC60 for comparison. All the samples were stored at 4°C under argon.

The morphology of the liposomes was observed under a transmission electron microscope (TEM-100X II, Electron, Japan). The particle size and Zeta potential of the liposomes were analyzed by a Malven Zetasizer (Zetasizer 3000HS, Malven Instruments, Germany).

MTT Assay. L-929 mouse fibroblasts were selected for the MTT cytotoxicity assay. After cell thawing and passage, the logarithmic growth cells were selected and digested by trypsin to prepare the cell suspension at a concentration of $1\sim10\times10^3$cells/mL. The cells suspension (100 μL) were seeded into 96-well plates and cultivated in a CO_2 incubator (Thermo Forma) at 37°C in a 5% CO_2/95% humidified air atmosphere. After cell adherence, the culture medium was replaced by 100 μL RPMI1640 medium (negative group), 90 μL RPMI1640 medium plus 10 μL TMCs-coated or uncoated CLs (test groups, namely TMC20 group, TMC40 group, TMC60 group, and uncoated group) or 100 μL DMSO (positive group), respectively. For each sample, 3–5 wells were prepared. After 48 h incubation, the cells were washed gently twice with PBS of pH 7.4. Totally, 10 μL MTT (5 g/L) was then added to each well and the cultures were incubated for an additional 4 h. DMSO (100 μL) was subsequently added to each well to dissolve the MTT formazan purple crystals after the supernatant fluid in each well was blotted. Absorbency (A) of the solutions was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) Reader [DG5031, ACON Biotech (Hangzhou, China)].

Cytotoxicity Evaluation. The relative growth rate (RGR) was used to evaluate cytotoxity. RGR was calculated using the following formula: $RGR = (A_{\text{test group}}/A_{\text{negative group}}) \times 100\%$. The standard of evaluation was in accordance with the relationship between cell RGR and cytotoxicity grade in the XXII Pharmacopoeia of the United States (USP) as depicted in Table I. Grade 0 and 1 are considered noncytotoxicity, while Grade 2 is mild cytotoxicity which should be combined with cell morphology to evaluate the cytotoxicity. Grades 3 and 4 both show moderate cytotoxicity, suggesting the incompatibility of the materials. If the RGR was in Grade 2, the cell morphology change was observed

Table I. The Relationship Between Cell RGR and Cytotoxicity Grade in USP

RGR(%)	Cytotoxicity grade
≥100	0
≥80	1
≥50	2
≥30	3
≥0	4

under an inverted phase contrast microscope (Olympus CK40-SL, Japan) to assess the cytotoxicity. The cells with good spindle or scalenous triangle shape and promising attachment are considered noncytotoxicity, the cells with promising attachment with minor circular-shrinkage are considered mild cytotoxicity, and dead suspension cells can be observed occasionally. As for the moderate cytotoxicity, the cells are with undesirable attachment and severe circular-shrinkage up to 1/3, and dead suspension cells can be seen largely.

In Vivo Tumor Angiogenesis Targeting of TMC60-Coated DOXL

Preparation of DOXL Coated by TMC60. DOXL were prepared by transmembrane ammonium sulfate gradients method described in the earlier study.[12] The homogenized DOXL suspension was drop wise added at the rate of 1 mL/min into the TMC60 water-solution with the optimal concentration screened by the above cytotoxicity evaluation under magnetic agitation of 30 rpm. The volumetric ratio of TMC60 solution and DOXL suspension was 4 : 1.[19] After the addition, agitation was kept for another 10 min to obtain TMC60-coated DOXL. The samples were stored at 4°C under argon.

The morphology, particle size, and Zeta potential of the liposomes were observed. G-50 Sephadex column (1.6 cm ×20 cm) was used to separate free DOX from the liposomes. The entrapment rate (ER%) of the liposomes was determined by a high-performance liquid chromatography (HPLC) using a UV-visible detector (254 nm for DOX, Agilent 1100).

***In Vivo* Tumor Angiogenesis Targeting.** H_{22} cells were chosen as cell samples in the study.[20] Experiments were carried out in male Kunming species mice. The animals were housed in single cages with free access to tap water and standard laboratory food throughout the experiments. FITC-Dextran (50 mg/mL) was injected via tail vein to locate the tumor angiogenesis. Laser confocal microscope (Leica TCS SP2, Germany) was applied to observe the morphology of tumor angiogenesis and the ELISA Reader was used to investigate the fluorescence intensity.

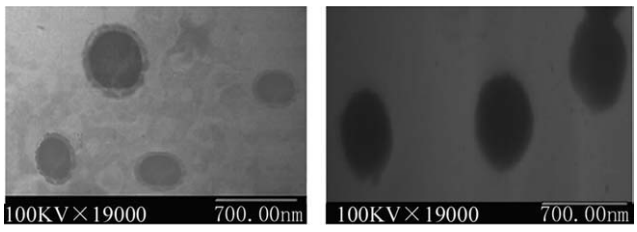

Figure 1. TEM images of liposomes prepared with TMC-coating (A) and without TMC-coating (B) (×19,000).

Table II. Mean Diameter and Zeta Potential of the Liposomes with or without TMC-Coating

Liposomes	Mean size (nm)	Zeta potential (mV)
Without TMC-coating	160.5 ± 6.7	−10.3 ± 0.2
0.2% TMC20-coated	213.6 ± 8.2	7.3 ± 0.2
0.2% TMC40-coated	218.0 ± 5.2	10.2 ± 0.4
0.2% TMC60-coated	210.8 ± 5.6	16.9 ± 0.4
0.15% TMC60-coated	202.4 ± 9.7	16.0 ± 0.2
0.1% TMC60-coated	193.9 ± 4.8	15.5 ± 0.5
0.07% TMC60-coated	186.1 ± 5.8	14.2 ± 0.2
0.05% TMC60-coated	178.7 ± 6.3	13.0 ± 0.6
0.02% TMC60-coated	171.6 ± 7.5	11.8 ± 0.5

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 seeding in abdominal cavity of the other healthy mice for 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.

Forty healthy mice were randomly divided into four testing groups with ten mice per group. Totally, 0.2 mL of neoplastic cell suspension was injected into the right limb armpit of each mouse. The whole injection process was completed in one hour. After 7 days, PS, free DOX solution, DOXL or TMC60-coated DOXL was injected via tail vein with the DOX amount of 5 mg/kg. Seven days after the injection, FITC-Dextran (50 mg/mL) 0.2 mL was injected via tail vein. Fifteen minutes later, the mice were sacrificed by cervical vertebra dislocation. The tumors were stripped and weighed. 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 PS, and treatment group was injected with free DOX, DOXL, and TMC60-coated DOXL, respectively. Then the tumors were fixed by immersion in 4% paraformaldehyde PBS overnight and dehydrated by immersion in 20% sucrose PBS for 2 days. The in situ fluorescence in the frozen sections was observed to evaluate the morphology of tumor angiogenesis. The fluorescence intensity was determined in the supernatant after the tumors were homogenized with PS (0.5 g/mL) to assess the tumor angiogenesis targeting of TMC60-coated DOXL.

Statistical Analysis

Data were expressed as the mean value ± SD. To determine the significant difference between different experimental groups, non-parametric 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, Chicago, IL).

RESULTS AND DISCUSSION

Properties of the TMCs-Coated CLs *In Vitro*

Typical TEM images of the liposomes with and without TMC-coating are shown in Figure 1(A, B), clearly exhibiting the well-defined TMC-coating layer. The mean size and Zeta potential are listed in Table II. It was found that the mean size was increased after TMC-coating, and DQ of TMC had no

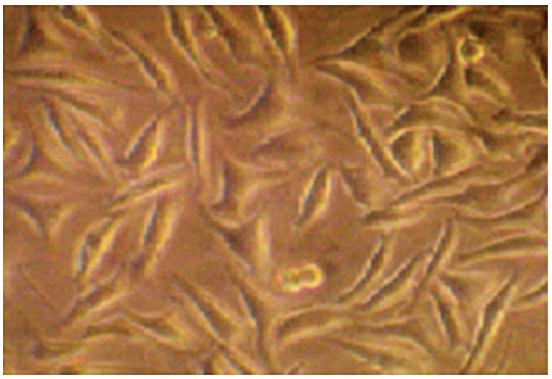

Figure 2. Photo of normal L-929 mouse fibroblasts (×10). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significant effect on the mean size at the same coating concentration. However, when the DQ was unchanged (TMC60), the mean size was increased with increasing TMC coating concentration, showing more TMC attached on the liposomes surface resulting in the higher Zeta potential. After TMCs coating, the Zeta potential of the liposomes was changed from negative to positive. Furthermore, with the increase of the DQ and concentration of TMCs, the absolute value of Zeta potential was increased accordingly.

Cytotoxicity of TMCs-Coated CLs

Cationic liposome is likely to be an effective tumor targeting delivery system. Its *in vivo* application has been, however, hindered by the toxicity associated with its formulations. The toxic effect is mainly determined for the free liposomes and cationic materials. As for the free liposomes, low toxicity can be obtained by purification of the polymers and addition of colipids or copolymers, such as cholesterol used in this study. In other related studies, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and dimethyl dioctadecylammonium bromide (DDAB) are the main cationic materials used to prepare CLs. Their cytotoxicity was increased significantly with the increased amount of the positive charge materials in the formulation.[21,22] Cationic polysaccharides display high biodegradation and low toxicity, and may be widely used in the pharmaceutics as idea cationic materials. Among them, CS water-soluble derivatives, such as TMCs, are the typical classes. However, the toxicity of cationic polysaccharides combined with free liposomes has not been studied yet. Therefore, it is necessary to study the cytotoxicity of CLs coated by TMCs to link the biocompatibility of the positive charge components for CLs with promising tumor vascular targeting ability.

Combined with the easy subcultivation and morphological observation, L-929 mouse fibroblasts are usually applied in the toxicity evaluation of biomaterials. In Figure 2, a photo of normal L-929 mouse fibroblasts is depicted, which belonged to adherent cells with spindle, scalenous triangle or fan-shape morphology and vortex, radial, or palisaded growth characteristics.

Table III shows the values of A, RGR, and cytotoxicity grade of various materials. The results clearly demonstrated noncytotoxicity of uncoated liposomes and TMC20-coated CLs with TMC20-coating concentration as high as 0.2%. TMC40 with 0.2% concentration showed grade two cytotoxicity and the cell morphology in Figure 3(B) exhibited minor circular-shrinkage when compared with that of the negative control group in Figure 3(A), suggesting mild cytotoxicity. As for TMC60 with concentration of 0.2%, the cytotoxicity grade was four, the same as that of the positive group, suggesting the coating concentration of TMC60 should be decreased in order to enhance the biosafety when applied in CLs. The results proved that the cytotoxicity of TMC-coated CLs had close relationship with the Zeta potential of the CLs, which was influenced by both the DQ and concentration of TMCs.

The A, RGR and cytotoxicity grade of TMC60 were determined with the coating concentration of 0.15, 0.1, 0.07, 0.05, and 0.02%, respectively and the results are listed in Table IV. For the 0.15% of TMC60, the cytotoxicity grade was three, showing moderate cytotoxicity. The cytotoxicity grade was two when the concentration was between 0.05 and 0.1% and the cell morphology was further observed [Figure 3(C–E)]. Although the cell number was reduced and the circular-shrinkage was observed under the effect of TMC60 with the concentration of 0.05–0.1%, the cytotoxicity was still mild with promising cell attachment when compared to the negative group. There was no significant difference among the various concentration group. As for TMC60 with 0.02% coating concentration, the cytotoxicity grade was one, showing noncytotoxicity. Taking both the cytotoxicity and the effectiveness of the coating process into considerration, the optimal coating concentration of TMC60 was determined as 0.1%. In the positive group, the normal cell morphology was damaged

Table III. A, RGR, and Cytotoxicity Grade of the TMCs-Coated Liposomes with TMCs Coating Concentration of 0.2% (w/w) on L-929 Mouse Fibroblasts

Group	Concentration (w/w, %)	A	RGR	Cytotoxicity grade
TMC20	0.2	1.451 ± 0.059	88.64	1
TMC40	0.2	1.097 ± 0.019	67.01	2
TMC60	0.2	0.49 ± 0.028	29.93	4
Uncoated		1.649 ± 0.035	100.7	0
Negative		1.637 ± 0.064	100	0
Positive		0.070 ± 0.004	4.28	4

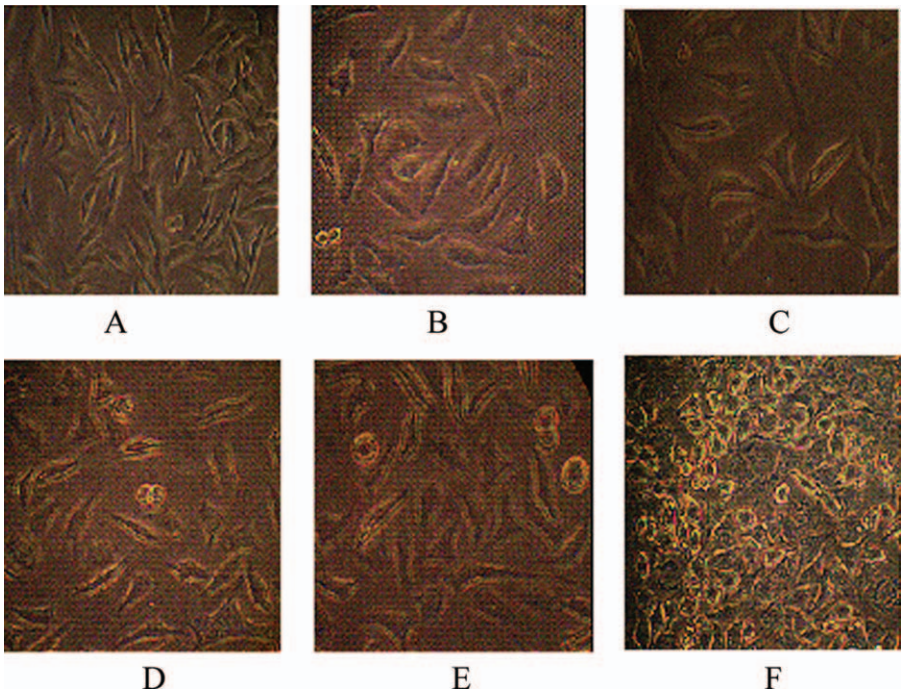

Figure 3. Photos of cell morphology of negative control group (A), different test groups with the cytotoxicity grade of two (B–E), and positive control group (F). (A) Negative control group, (B) CLs coated by 0.2%TMC40, (C) CLs coated by 0.05%TMC60, (D) CLs coated by 0.07%TMC60, (E) CLs coated by 0.1%TMC60, (F) positive control group.

completely with major circular-shrinkage and many dead suspension cells observed.

Properties of TMC60-Coated DOXL

The morphology of TMC60-coated DOXL was similar to that shown in Figure 1(A) with distinct coating layer. The average size was 218.8 ± 5.6 nm and Zeta potential was 16.2 ±0.2 mV. Compared with those of the blank TMC60-coated CLs, DOX-loading increased the size a little while had no influence on the morphology and Zeta potential. The ER% was 68.5 ± 1.6% and 64.7 ± 1.4% before and after TMC60-coating, suggesting TMC60-coating had no significant effect on the ER% of DOXL.

In Vivo Tumor Angiogenesis Targeting Ability of TMC60-Coated DOXL

The growth, invasion, and metastasis of tumor cells all depend on the formation of tumor angiogenesis. Therefore, inhibition of tumor angiogenesis formation which leads to the cutting-off of the nutritional pathway for tumor cells could effectively inhibit the proliferation of tumor cells. The direct proportional relationship between the tumor angiogenesis targeting ability and the positive surface charge of the cationic liposomes had been reported.[23] With the increase of CLs positive charge, the tumor targeting ability was enhanced. In our earlier study, it was found that the tumor inhibition rate of TMCs-coated DOXL was increased with the DQ increase of TMCs, which further verified the importance of positive charge in the targeting ability of CLs. According to the results of above cytotoxicity study *in vitro*, the cytotoxicity of 0.2% TMC40 and 0.1% TMC60 were both mild. Although TMC60 had better tumor inhibition effect than TMC40 at the same concentration, 0.2% TMC40 was also a potential material with promising tumor angiogenesis targeting ability. The comparison study between the two kinds of TMC with different concentrations is

Table IV. Influence of Liposomes Coated by TMC60 with Various Concentrations on Mouse Fibroblasts Cell L-929

Groups	Concentration (w/w, %)	A	RGR	Cytotoxicity grade
	0.02	1.503 ± 0.034	94.53	1
	0.05	1.251 ± 0.016	78.68	2
TMC60	0.07	1.248 ± 0.030	78.49	2
	0.1	0.996 ± 0.043	62.64	2
	0.15	0.767 ± 0.027	48.24	3
Negative		1.590 ± 0.047	100	0

Table V. The Inhibition Rate of Transplanted H_{22} Solid Tumor in Mice Treated by PS, free DOX solution, DOXL, and TMC60-Coated DOXL, respectively, ($n = 10$, $\bar{x} \pm s$)

Groups	Weight of tumor (g)	Tumor inhibition rate (%)
PS	1.9311 ± 0.006	-
DOX	1.7622 ± 0.041	8.75
DOXL	1.2575 ± 0.041	34.88
TMC60-coated DOXL	0.8986 ± 0.008	53.47[a]

[a]Note: Compared with free DOX solution and DOXL, $P < 0.05$

undergoing in our lab. In the present study, TMC60 with 0.1% coating concentration screened by the above cytotoxicity evaluation was chosen as the coating material in the experiment of *in vivo* tumor angiogenesis targeting ability.

Forty healthy mice were used in the study, and the mortality was zero through the whole process. The tumor weight and inhibition rate of various DOX preparations are listed in Table V. Compared with free DOX solution and DOXL, TMC60-coated DOXL had the minimum tumor weight, and enhanced the tumor inhibition rate significantly, which was consistent with the results in our earlier studies.[12]

As for the effective treatments of anti-angiogenesis formation, there are always significant changes in the morphology, arrangement, and density of neoplasm vascularity besides the reduction of tumor volume and weight. FITC-Dextran, a fluorescent polymer, can attach to the surface of endothelial cells without uptake and pass through endothelial junction in a short time after intravenous injection. Therefore, it can be applied in labeling the vascularity formation *in vivo*.[24] Meanwhile, the FITC-Dextran amount attached to the surface of the tumor vessel will be decreased with the reduction of tumor vessel density. Therefore, the determination of FITC-Dextran fluorescence intensity in tumor tissue can determine the tumor vessel density.

Under the laser confocal microscope, the histological section images of H_{22} transplantation tumor of mouse hepatoma are displayed in Figure 4. Among them, the tumor vessels of PS control group showed the following properties: the angiogenesis

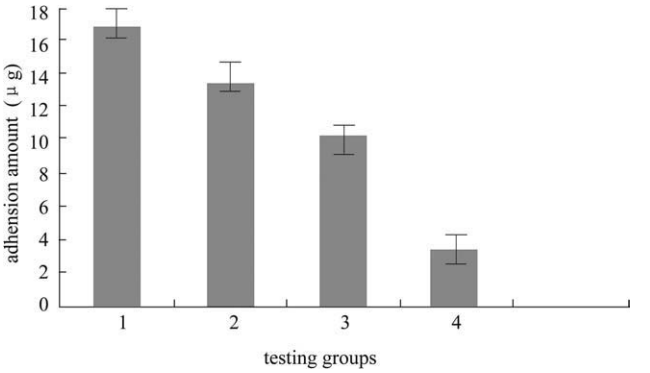

Figure 5. The FITC-Dextran adhesion amount in the tumor tissue of the testing groups, 1-PS group; 2-free DOX group; 3-uncoated DOXL group; 4-TMC60-coated DOXL group.

was immature with maldistribution, distortion, expansion, mutual adhension and intersection, and artery-vein access without proper anastomosis. DOX and DOXL group both showed reduced vessel number with less intersection and the diameter of vessels were uneven. Compared with the above groups, TMC60-coated DOXL group displayed the promising vessels morphology with further reduced number, uniform distribution and the least intersection. The results proved that TMC60-coated DOXL could significantly regulate and improve the microvascular structure, morphology and number, and had notable angiogenesis targeting ability.

The FITC-Dextran adhesion amount in the tumor tissue of each group as depicted in Figure 5 showed that TMC60-coated DOXL group had the least adhesion amount when compared with the other groups. The results further proved that TMC60-coated DOXL could significantly reduce the density of angiogenesis and had notable anti-tumor angiogenesis effect.

CONCLUSIONS

In this study, the blank CLs were prepared by TMC-coating and their cytotoxicity on L-929 mouse fibroblasts was evaluated by MTT assay. With the increase of coating concentration and DQ of the TMCs, the cytotoxicity of the CLs was increased accordingly. TMC20 with the maximum testing concentration of 0.2% showed noncytotoxicity, TMC40 of 0.2% and TMC60 of 0.1% were both with mild cytotoxicity, which should be ideal cationic

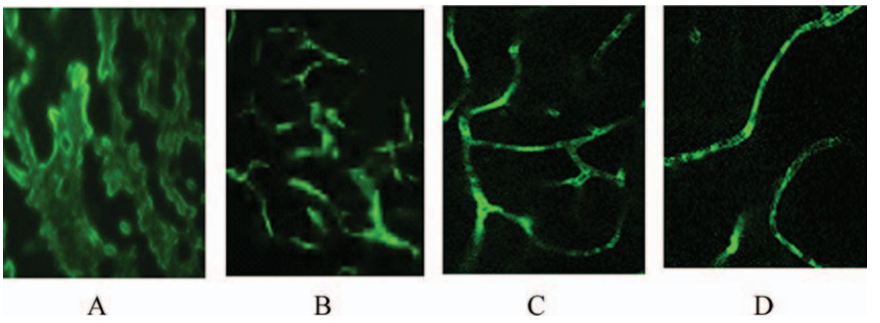

Figure 4. The fluorescence in tumor tissue frozen section of each experimental group, (A) PS group, (B) free DOX group, (C) uncoated DOXL group, (D) TMC60-coated DOXL group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

lipid coating materials. Compared with those of free DOX and uncoated DOXL, the *in vivo* antitumor activity and tumor angiogenesis targeting ability of DOXL coated by 0.1% TMC60 were both enhanced significantly. The results verified that CLs could be easily prepared by TMCs-coating with promising safety and notable enhancement for tumor angiogenesis targeting. The mechanisms of enhancement by TMCs-coated CLs are being further studied.

REFERENCES

1. Marcus, S. S.; Sebastian, S.; Senat, K.; Birgitta, S.; Brita, S.; Michael, T.; Uwe, M.; Kurt, N.; Marc, D. *Clin. Cancer Res.* **2003,** *9*, 2335.
2. Senat, K.; Alexander, W.; Martin, E. E.; Marcus, S. S.; Sascha, A. P.; Birgitta, S.; Brita, S.; Michael, T.; Uwe, M.; Kurt, N.; Marc, D. *Int. J. Cancer* **2003,** *105*, 561.
3. Sebastian, S.; Martin, E. E.; Birgitta, S.; Brita, S.; Michael, T.; Uwe, M.; Marc, D. *Int. J. Cancer* **2004,** *110*, 117.
4. Kalra1, A. V.; Campbell, R. B. *Pharm. Res.* **2006,** *12*, 2809.
5. Schmit-Sody, M.; Stieth, S.; Krasnici, S.; Sauer, B.; Schulze, B.; Teifel, M.; Michaelis, U.; Naujoks, K.; Dellian, M. *Clin. Cancer Res.* **2003,** *9*, 35.
6. Thurston, G.; McLean, J. W.; Rizen, M.; Baliuk, P.; Haskell, A.; Murphy, T. J.; Hanahan, D.; Mcdonald, D. M. *J. Clin. Invest.* **1998,** *101*, 1401.
7. Krasnici, S.; Werner, A.; Eichhorn, M. E.; Schmitt-Sody, M.; Pahernik, S.; Sauer, B.; Schulze, B.; Teifel, M.; Michaelis, U.; Naujoks, K.; Dellian, M. *Int. J. Cancer* **2003,** *5*, 561.
8. Augustin, H. G.; Kozian, D. H.; Johson, R. C. *Bioassays* **1994,** *16*, 901.
9. Mounkes, L. C.; Zhong, W.; Cipres-Palacin, G.; Heath, T. D.; Debs, R. J. *Biol. Chem.* **1998,** *273*, 164.
10. Ran, S.; He, J.; Huang, X. M.; Soares, M.; Scothom, D.; Thorpe, P. E. *Clin. Cancer Res.* **2005,** *11*, 1151.
11. He, W.; Guo, X. X.; Xiao, L. H.; Feng, M. *Int. J. Pharm.* **2009,** *382*, 334.
12. Guo, X. X.; He, W.; Wang, X. Q.; Liu, X. Y.; Hu, X. M. *J. Appl. Polym. Sci.* **2011,** *121*, 2149.
13. Dass, C. R. *J. Pharm. Pharmacol.* **2002,** *54*, 593.
14. Lv, H. T.; Zhang, S. B.; Wang, B.; Cui, S. H.; Yan, J. J. *Controlled Release* **2006,** *114*, 100.
15. Liu, W. G.; Yao, K. D. *J. Controlled Release* **2002,** *83*, 1.
16. Florea, B. I.; Thanoum, M.; Junginger, H. F.; Borchard, G. J. *Controlled Release* **2006,** *110*, 353.
17. Snyman, D.; Hamman, J. H.; Kotze, J. S.; Rollings, J. E.; Kotze, A. F. *Carbohydr. Res.* **2001,** *50*, 145.
18. Hamman, J. H.; Kotze, A. F. *Drug Dev. Ind. Pharm.* **2001,** *27*, 373.
19. Wei, N.; Lu, B. *Acta Pharm. Sinica* **2003,** *38*, 53.
20. Schiffelers, R. M.; Koning, G. A.; Hagen, T.L. M.; Fens Marcel, H.A. M.; A Schraa, S. J.; Janssen Adrienne, P.C. A.; Kok, R. J.; Molema, G.; Storm, G. J. *Controlled Release* **2003,** *91*, 115.
21. Dass, C. R. *Int. J. Pharm.* **2003,** *267*, 1.
22. Wu, J.; Lee, A.; Lu, Y. H.; Lee, R. *Int. J. Pharm.* **2007,** *337*, 329.
23. Campbell, R. B.; Fukumura, D.; Brown, E. B.; Mazzola, L. M.; Izumi, Y.; Jain, R. K.; Torchilin, V. P.; Munn, L. L. *Cancer Res.* **2002,** *62*, 6831.
24. Zhao, W.; Ma, H. L.; Qi, X. R. *Acta. Pharm. Sinica* **2007,** *42*, 982.