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Preclinical evaluation of thermosensitive poly(*N*-(2-hydroxypropyl) methacrylamide mono/dilactate)-grafted liposomes for cancer thermochemotherapy

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ABSTRACT

Thermosensitive liposomes grafted with cholesterol-conjugated poly(*N*-(2-hydroxypropyl) methacrylamide mono/dilactate) (chol-pHPMAlac) have been developed for heat-induced release of doxorubicin (DOX). These liposomes release DOX completely during mild hyperthermia, but their interaction with blood cells and cancer cells has not been studied. Following intravenous administration, liposomes may interact with plasma proteins and various types of cells (e.g., endothelial cells, platelets, and macrophages), which would reduce their disposition in the tumor stroma. Interaction between liposomes and platelets may further cause platelet activation and thrombosis, which could lead to vascular occlusion and thromboembolic complications. The aim was to investigate DOX release kinetics in the presence of serum, stability, in vitro uptake by and toxicity to cancer cells and somatic cells, and platelet activating potential of the chol-pHPMAlac liposomes. DOX release was determined spectrofluorometrically. Liposome stability was determined in buffer and serum by dynamic light scattering and nanoparticle tracking analysis. Association with/uptake by and toxicity of empty liposomes to AML-12, HepG2 (both hepatocyte-derived cancer cells), RAW 264.7 (macrophages), and HUVEC (endothelial) cells was assayed in vitro. Platelet activation was determined by analysis of P-selectin expression and fibrinogen binding. DOPE:EPC liposomes (diameter = 135 nm) grafted with 5% chol-pHPMAlac (cloud point (CP) = 16 °C; M_n = 8.5 kDa) released less than 10% DOX at 37 °C in 30 min, whereas complete release took place at 47 °C or higher within 10 min. The size of these liposomes remained stable in buffer and serum during 24 h at 37 °C. Fluorescently labeled but DOX-lacking chol-pHPMAlac-liposomes exhibited poor association with/uptake by all cells under investigation, were not cytotoxic, and did not activate platelets in both buffered solution and whole blood. In conclusion, thermosensitive chol-pHPMAlac-grafted liposomes rapidly release DOX during mild hyperthermia. The liposomes are stable in a physiological milieu, are not taken up by cells that are encountered in an in vivo setting, and are non-antagonistic towards platelets. Chol-pHPMAlac-grafted liposomes are therefore good candidates for DOX delivery to tumors and temperature-triggered release in tumor stroma.

1. Introduction

Liposomes for systemic cancer therapy are generally designed for stability in the circulation and therefore retain their cargo until uptake by cells. Consequently, drug release from these liposomes is slow and uncontrolled, resulting in sublethal concentrations of free drug in the tumor and poor therapeutic efficacy (Bandak et al., 1999; Landon et al., 2011). This problem can be resolved with temperature-sensitive liposomes (TSLs) that release water-soluble anti-cancer drugs at a fast rate upon a heat stimulus (Limmer et al., 2014; Needham et al., 2000; Tagami et al., 2012). Numerous studies have demonstrated rapid intravascular release of doxorubicin (DOX) from thermally triggered

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TSLs, leading to high intravascular drug concentrations and enhanced drug penetration into tumor tissue (Kong et al., 2000; Landon et al., 2011; Manzoor et al., 2012; Needham et al., 2000). Under mild hyperthermia, the DOX-TSLs yielded 20–30 times greater DOX deposition in tumor tissue compared to free DOX administration, that in turn translated to improved tumor cell-killing efficacy (Kong et al., 2000; Yarmolenko et al., 2010). The application of mild hyperthermia locally has two other important advantages. Firstly, hyperthermia increases intratumoral blood flow and vascular permeability (Gaber et al., 1996), enabling more extensive liposome extravasation into the tumor and thus higher local drug concentration (Kong et al., 2001; Matteucci et al., 2000). Secondly, hyperthermia exacerbates the cytotoxicity of some chemotherapeutics (Hahn et al., 1975; Herman, 1983; Landon et al., 2011), including DOX (Liu et al., 2001).

Instead of using phospholipids that undergo phase transition at a temperature (T_m) slightly above body temperature (van Raath et al., 2016), second-generation TSLs are grafted with thermolabile polymers that destabilize the phospholipid bilayer at elevated temperatures and induce release of liposomal content (van Elk et al., 2014). Temperature-sensitive polymers composed of *N*-(2-hydroxypropyl)methacrylamide mono/dilactate exhibit tuneable critical solution temperature behavior within the physiologically relevant temperature window. These polymers are soluble in aqueous solution at a low temperature but dehydrate and aggregate when heated above their cloud point (CP). As proof-of-concept, polymers of *N*-(2-hydroxypropyl)methacrylamide mono/dilactate were synthesized with a cholesterol anchor (chol-pHPMAlac) and incorporated into DOX-encapsulating liposomes. The chol-pHPMAlac TSLs released DOX at mild hyperthermia in buffered aqueous solution (van Elk et al., 2014). The next step towards clinical translation is to determine the interaction between the chol-pHPMAlac TSLs and physiologically relevant cell types such as blood cells, endothelial cells, immune cells, and cancer cells.

Previous translational studies on thermosensitive liposome-cell interactions have demonstrated some association with blood cells, endothelial cells, immune cells, primary hepatocytes (Heger et al., 2009b; van Raath et al., 2016), and cancer cells (Broekgaarden et al., 2014). The liposome-cell interactions are, however, mostly dependent on liposome surface properties and size, and not the fact that they are thermosensitive. For example, van Raath et al. observed some association between DPPC:DSPE-PEG (96:4) liposomes (T_m of 42.3 °C) and hamster and human platelets, irrespective of their activation state. Moreover, the liposomes associated with hamster erythrocytes (in vitro and in vivo) and human erythrocytes (in vitro), although these findings may be ascribable to the leakage and subsequent uptake of the fluorescent probe by the cells. Liposomes composed of DSPC:DPPC:DSPE-PEG (85:10:5 and 46:50:4, neutral surface charge), DPPC:DPPE:DSPE-PEG (46:50:4, neutral surface charge), and DPPC:DMPA:DSPE-PEG (46:50:4) did not notably associate with resting and activated hamster platelets (Heger et al., 2009a). Moreover, thermosensitive DPPC:DSPE-PEG (96:4) liposomes do not induce hemolysis, are minimally taken up by cultured endothelial cells and hepatocellular carcinoma (HepG2) cells, but are endocytosed by mouse RAW 264.7 macrophages (van Raath et al., 2016). Finally, the extent of uptake by cancer cells is dependent on particle size (Broekgaarden et al., 2014). Given the presence of polymer with presently unknown bio-interactive properties on the surface chol-pHPMAlac TSLs, the particle-cell interactions may differ relative to earlier findings.

The aim of this follow-up study was therefore to investigate parameters that are relevant for in vivo implementation of the chol-pHPMAlac DOX-TSLs, which included (1) DOX release kinetics in the presence of plasma proteins; (2) stability in a quasi-biological environment; (3) association or uptake of chol-pHPMAlac TSLs by tumor-comprising cells; (4) cytotoxicity of empty chol-pHPMAlac TSLs to cancer cells and somatic cells; and (5) platelet-activating potential of the chol-pHPMAlac TSLs.

2. Materials and methods

2.1. Materials

Thiocholesterol, *N,N,*-azobisisobutyronitrile (AIBN), Triton X-100 (TX-100), formaldehyde, sodium dodecyl sulfate (SDS), Dulbecco's modified Eagle's medium (high glucose) (DMEM), fetal bovine serum (FBS), prostacyclin, and antibiotic/antimycotic solution (100×) were purchased from Sigma-Aldrich (St. Louis, MO). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 3β-[N-(N′,N′-dimethylaminoethane)-carbimoyl] cholesterol (DC-cholesterol), and L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rho-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Egg phosphocholine (EPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)] (DSPE-PEG, average polymer mass of 2000 Da) were obtained from Lipoïd (Ludwigshafen, Germany). Doxorubicin-HCl was purchased from Guanyu Bio-technology (Xi'an, China). EGM-2 kit + EBM medium and human umbilical vein endothelial cells (HUVECs) were acquired from Lonza (Basel, Switserland). RAW 264.7 and HepG2 cells were obtained from ATCC (Manassas, VA). AML-12 cells were provided by Dr. Riekelt Houtkooper (Academical Medical Center, Amsterdam). Lipofectamine 2000 was purchased from Invitrogen/Life Technologies (Carlsbad, CA). APC-conjugated mouse anti-human CD42b antibodies (clone HIP1) and PE-conjugated mouse anti-human P-selectin antibodies (clone AK-4) were obtained from BD Biosciences (Franklin Lakes, NJ). Polyclonal FITC-conjugated rabbit anti-human fibrinogen antibodies were purchased from Dako (Glostrup, Denmark). Thrombin receptor activator peptide 6 (TRAP-6) was obtained from Bachem (Bubendorf, Switzerland). 1,4-Dioxane was purchased from Biosolve (Valkenswaard, the Netherlands). All other chemicals were of analytical grade.

2.2. Synthesis of HPMA mono/dilactate polymers with cholesterol anchor

2-Hydroxypropyl methacrylamide (HPMA), HPMA monolactate, and HPMA dilactate were synthesized as described previously (Neradovic et al., 2003). HPMA mono/dilactate polymers with a cholesterol anchor (chol-pHPMAlac) were synthesized according to a previously reported method (Paasonen et al., 2007; van Elk et al., 2014). Briefly, HPMA monolactate/dilactate (8 mmol) was dissolved in distilled 1,4-dioxane at a concentration of 100 mg/mL and at a monolactate:dilactate molar ratio of 50:50 or 100:0. AIBN was used as initiator at a molar ratio of 500:1 (monomer/AIBN). The molar ratio of thiocholesterol (chain transfer agent) and the HPMA monomers was 1:50. Three nitrogen/vacuum cycles were applied to remove oxygen and the polymerization reaction was performed for 24 h at 70 °C. The obtained polymers were precipitated in diethyl ether and the supernatant was discarded after centrifugation (3000 × *g*, 15 min at 4 °C). The polymers were dried overnight in a vacuum oven at 25 °C and stored at −20 °C until further use. Obtained polymers are abbreviated as: chol-pHPMAlac(x-y), in which x-y refers to the relative amount of HPMA monolactate (x) and HPMA dilactate (y), respectively.

2.3. Polymer characterization

The copolymer composition of chol-pHPMAlac was determined by ^{1}H-NMR (Gemini 300 MHz spectrometer, Varian, Palo Alto, CA) in $(CD_3)_2SO$. The ratio of HPMA monolactate/HPMA dilactate (ML/DL) was determined from the integral of the peak at 5.0 ppm (CO-C*H*(CH_3)-O) divided by the integral of the peaks at 4.1 and 4.2 ppm (CO-C*H*(CH_3)-OH).

The cloud point (CP) of chol-pHPMAlac was determined by light scattering at 650 nm (model UV-2450, Shimadzu Scientific Instruments, Kyoto, Japan). Chol-pHPMAlac was dissolved at 5 mg/mL in 120 mM ammonium acetate buffer, pH = 5.0. Samples were heated from 0 to

70 °C at a rate of 1 °C/min. The CP was defined as the onset temperature at which an increase in light scattering occurred.

The number average molecular weight (M_n) and weight average molecular weight (M_w) of chol-pHPMAlac were measured using gel permeation chromatography (GPC) using a refractive index detector, a Plgel 5-μm MIXED-D column, and PEGs with narrow molecular weights as standards. DMF containing 10 mM LiCl was used as eluent at an elution rate of 1.0 mL/min. The column temperature was maintained at 65 °C.

2.4. Preparation of doxorubicin-loaded liposomes

Three different liposomal formulations were prepared: temperature-sensitive polymer-coated liposomes composed of DOPE:EPC:chol-pHPMAlac(43-57) (70:25:5 molar ratio) and DOPE:EPC:chol-pHPMAlac (82-18) and a non-temperature sensitive formulation consisting of DOPE:EPC:DSPE-PEG2000 (70:25:5). The phospholipids and chol-pHPMAlac were dissolved in 5 mL chloroform (15 μmol phospholipids/mL). A lipid film was formed after evaporation of chloroform under reduced pressure and the remaining traces of chloroform were removed overnight under nitrogen flow. The lipid film was subsequently hydrated with 5 mL of 240 mM ammonium sulfate buffer (pH = 5.4) to a concentration of 15 μmol phospholipid/mL. The liposomal dispersion was extruded 2× through two 200-nm filters (GE Water & Process Technologies, Trevose, PA) and 8× through two 100-nm filters (LIPEX extruder, Northern Lipids, Burnaby, Canada). The extruded liposomes were dialyzed against 20 mM HEPES buffer, pH = 7.4, containing 8 g NaCl/L. The liposomes (3 mL) were loaded with doxorubicin (DOX, 5 mg/mL, 1.5 mL) for 4 h at 4 °C to obtain self-quenching DOX concentrations in the liposomes (van Elk et al., 2014). Free DOX and free chol-pHPMAlac were removed by ultracentrifugation (125,000 × *g* for 45 min at 4 °C). The liposome-containing pellet was resuspended in 3 mL of 20 mM HEPES buffer, pH = 7.4, and stored at 4 °C in the dark.

2.5. Preparation of fluorescent liposomes for cell association studies

Empty liposomes labeled with Rho-PE (DOPE:EPC:chol-pHPMAlac (43-57)/chol-pHPMAlac(82-18)/DSPE-PEG2000:Rho-PE, 70:25:5:0.1) were prepared as described in Section 2.4 with some modifications. The lipid film was hydrated with 5 mL phosphate buffered saline (PBS), and after extrusion the unbound chol-pHPMAlac was removed by ultracentrifugation (125,000 × *g* for 45 min at 4 °C). The liposomes were resuspended in 5 mL of PBS and stored at 4 °C. Cationic liposomes consisting of DPPC:DC-cholesterol:cholesterol:Rho-PE, 70:25:5:0.1 (referred to as DC-cholesterol liposomes) were prepared as described by Broekgaarden et al. (Broekgaarden et al., 2014) as a positive control formulation for the cellular association/uptake assays.

2.6. Liposome characterization

The size and the polydispersity index (PDI) of liposomes suspended in PBS were measured by dynamic light scattering (DLS, CGS-3 multi-angle goniometer, Malvern Instruments, Malvern, PA). Intensity correlation functions were measured using a wavelength of 632.8 nm, a scattering angle of 90°, and unimodal analysis. The size of the liposomes was also determined by nanoparticle tracking analysis (NTA) using a NanoSight LM10SH instrument with a 532-nm laser and an EMCDD camera (Malvern Instruments). The advantage of this technique is that NTA is able to track fluorescent particles in a medium that contains other (non-fluorescent) particular contaminants. NTA was therefore used to analyze particle size in FBS, which would not be possible by DLS. Liposomes were incubated in PBS or FBS at 37 °C for 1 or 24 h, after which the liposomes were diluted in PBS and measured for 60 s with a threshold of 4. The captured videos were analyzed with NTA 3.0 image analysis software (Malvern Instruments). The zeta (ζ) potential was determined in 20 mM HEPES buffer, pH = 7.4, by laser Doppler electrophoresis using a Zetasizer Nano-Z (Malvern Instruments).

Table 1
Characteristics of chol-pHPMAlac.

Copolymer composition[a] (monolactate:dilactate) (mol/mol)	Yield	M_n[b] (kDa)	M_w[b] (kDa)	PDI[b]	CP[c]
43:57	64%	8.5	12.5	1.5	16 °C
82:18	84%	9.0	14.0	1.6	42 °C

[a] Determined by ^{1}H-NMR
[b] Determined by gel permeation chromatography
[c] Determined by DLS at 650 nm

Table 2
Characteristics of temperature sensitive and non-temperature sensitive liposomes used in this study.

	Chol-pHPMAlac (43-57)	Chol-pHPMAlac (82-18)	DSPE-PEG2000
Size (PBS)[a]	135 ± 1 nm	136 ± 1 nm	135 ± 3 nm
PDI (PBS)[a]	0.06 ± 0.01	0.08 ± 0.03	0.09 ± 0.08
Size (PBS)[b] at 37 °C	656 ± 1 nm	135 ± 1 nm	138 ± 3 nm
PDI (PBS)[b] at 37 °C	0.61 ± 0.01	0.09 ± 0.03	0.08 ± 0.08
ζ-potential (20 mM HEPES, pH = 7.4)[c]	−5 ± 4 mV	−4 ± 2 mV	−7 ± 1 mV
ζ-potential (20 mM HEPES, pH = 7.4 + 0.8% NaCl)[c]	−0.3 ± 1.0 mV	1 ± 2 mV	−1 ± 2 mV

[a]Determined by DLS before the heating run
[b]Determined by DLS at 37 °C measured during a heating run from 10 to 60 °C at a heating rate of 1 °C/min
[c]Determined by laser Doppler electrophoresis

The total DOX concentration was determined after solubilization of the liposomes with TX-100 (0.1% final concentration) (van Elk et al., 2014) using fluorescence spectroscopy (λ_{ex} = 485 ± 5 nm, λ_{em} = 600 ± 5 nm, FLUOstar Optima, BMG Labtech, Ortenberg, Germany). Concentrations were calculated from the linear fit function of the DOX standard curve.

The time-based release of DOX was measured by the change in fluorescence intensity in time (λ_{ex} = 468 ± 5 nm, λ_{em} = 558 ± 5 nm, Fluorolog, Horiba Scientific, Edison, NJ). DOX-loaded liposomes (1 μL, 15 mM phospholipids) were added to 20 mM HEPES buffer (pH = 7.4, 1.6 mL) preheated to 37, 42, 47, 52, or 57 °C and the fluorescence intensity was measured in kinetics mode. TX-100 (10%, 16 μL) was added at the end of the experiment to solubilize the liposomes to determine the total amount of DOX present. The percentage DOX release was calculated using the following equation: $(I_t - I_0)/(I_{TX} - I_0) \times 100\%$, in which I_t is the fluorescence intensity at time t, I_0 the intensity at the start of the experiment, and I_{TX} the fluorescence intensity after addition of TX-100.

2.7. Cell lines and culture conditions

Human umbilical vein endothelial cells (HUVECs) were cultured in EGM 2 plus EBM medium. Murine RAW 264.7 macrophages were cultured in DMEM high glucose medium. Mouse AML-12 hepatocytes and human hepatocellular carcinoma HepG2 cells were cultured in William's E medium supplemented with 10% L-glutamine. The culture media were supplemented with 10% FBS, penicillin (100 IU/mL) (AML-12 and HepG2 cells only), streptomycin (100 μg/mL) (AML-12 and HepG2 cells only), and amphotericin B (0.25 μg/mL) (AML-12 and HepG2 cells only). Cells were cultured at standard conditions (37 °C, 5% CO_2, humidified atmosphere).

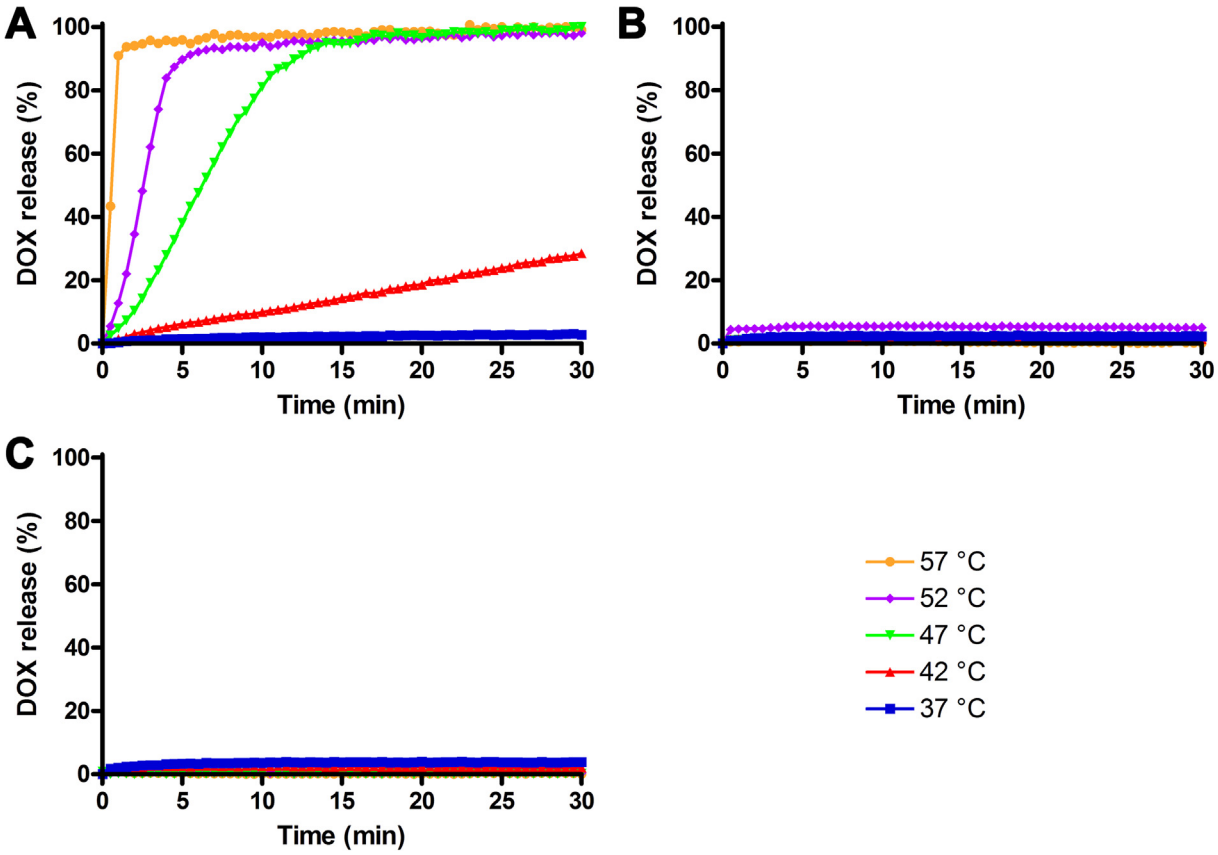

Fig. 1. Heat-induced release of DOX from DOPE:EPC (70:25) liposomes grafted with 5% chol-pHPMAlac(43-57) (A), chol-pHPMAlac(82-18) (B), and DSPE-PEG2000 (C). Release was measured in buffer (20 mM HEPES, 0.8% NaCl, pH = 7.4) and plotted relative to 100% release following TX-100 treatment (not shown). Representative traces are shown for N = 3 replicate experiments per temperature group.

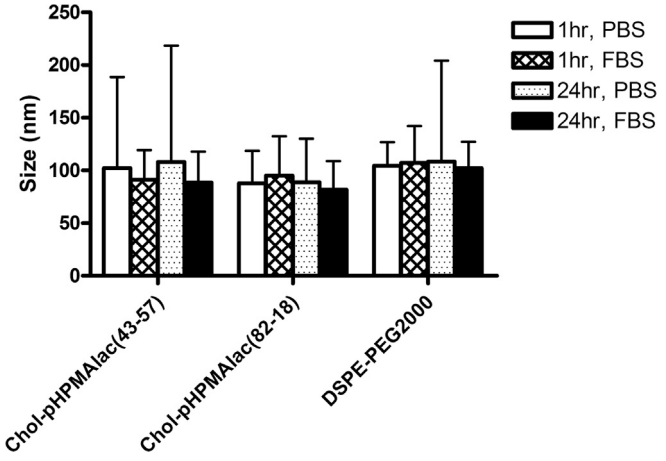

Fig. 2. Size of DOPE:EPC:Rho-PE liposomes (70:25:0.1) grafted with 5 mol% polymer following incubation at 37 °C in PBS or FBS for 1 or 24 h. Size was determined by NTA.

2.8. Cell association assays

For cell association studies, HUVECs, AML-12, HepG2, and RAW 264.7 cells were seeded in 96-wells plates at a density of 4.2×10^4 cells/well (90 μL, near confluence for HUVECs, HepG2, and RAW 264.7 cells, 60% confluence for AML-12 cells). After 4 h, the cells were incubated with 10 μL prediluted Rho-labeled liposomes (Section 2.5, 0–150 μM final phospholipid concentration, i.e., a clinically relevant concentration range) for 30 min or 24 h at standard culture conditions. Cells were washed 2× with PBS and lysed with 20% methanol and 0.1% SDS in PBS. The fluorescence intensity of Rho was measured at λ_{ex} = 550 ± 5 nm, λ_{em} = 600 ± 5 nm (FLUOstar Optima). Relative fluorescence intensities were plotted versus the phospholipid concentration of the liposomes. Moreover, liposome uptake by/association with cells was visualized by fluorescence- and phase contrast microscopy (BZ-9000, Plan Fluor 20× objective, Keyence, Osaka, Japan). Cells were cultured and incubated with Rho-liposomes as described above, washed 2× with PBS after 30 min incubation, and imaged (Texas Red filter set, λ_{ex} = 562 ± 20 nm, λ_{em} = 624 ± 20 nm).

2.9. Cell viability assay

Cell viability was determined with the MTS assay (CellTiter 96, Promega, Madison, WI) following incubation with liposomes or lipofectamine (positive control). HUVECs, AML-12, HepG2, and RAW 264.7 cells were seeded in 96-wells plates at a density of 2×10^4 cells/well (100 μL). After 24-hour incubation (confluence of ~70%), the medium was refreshed (90 μL/well) and 10 μL of the liposomal suspension or PBS (control) was added to a final phospholipid concentration ranging from 0 to 150 μM. Cells were incubated with the liposomes for 24 h. Hydrogen peroxide (H_2O_2, 10 μL, 30%) was added to several control wells to induce cell death. Subsequently, the medium was refreshed and 20 μL of CellTiter 96 was added per well. The plates were incubated at standard conditions for 4 h, after which absorbance was measured at 492 nm with a reference absorbance at 620 nm. The cell viability was calculated using the following equation: $(I_{sample} - I_{H2O2})/(I_{PBS} - I_{H2O2}) \times 100\%$ in which I_{sample} is the absorbance of the sample incubated with liposomes, I_{H2O2} the absorbance after adding hydrogen peroxide, and I_{PBS} the absorbance of the cells incubated with PBS.

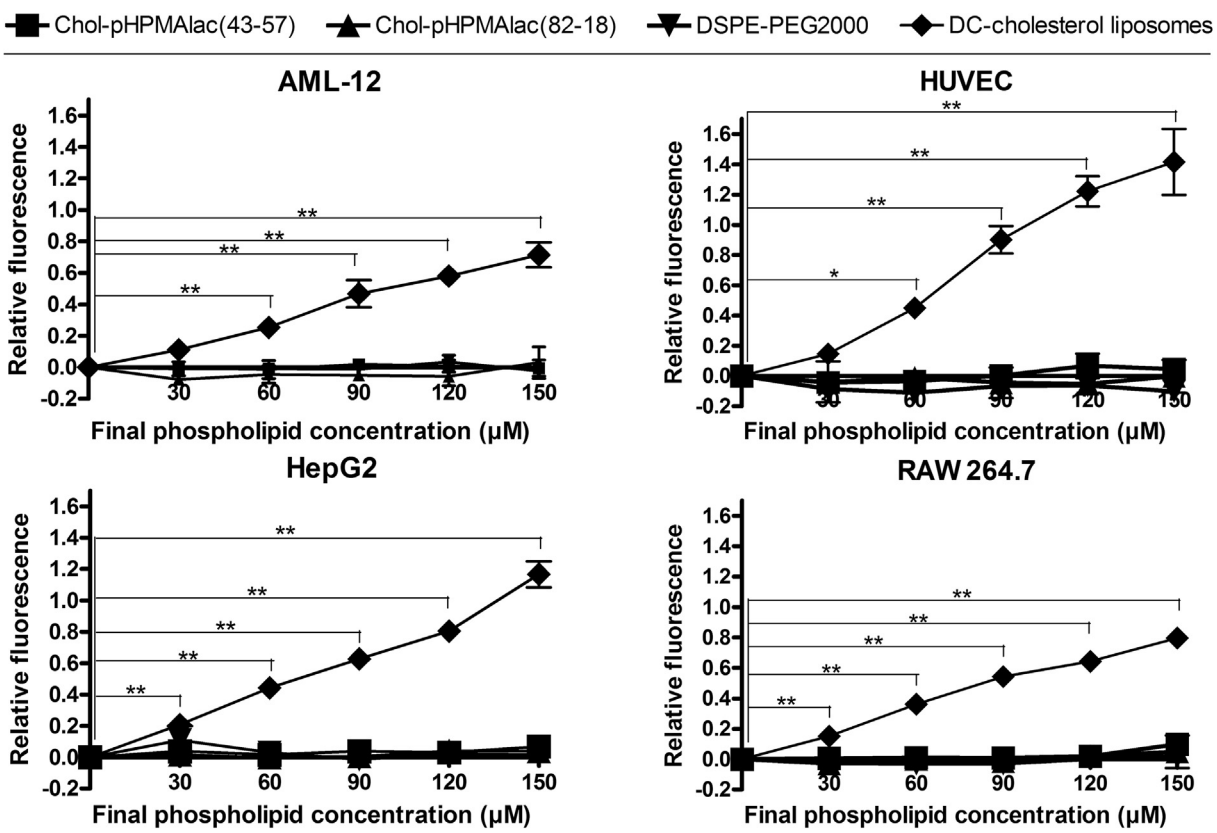

Fig. 3. Association between fluorescent DOPE:EPC:Rho-PE liposomes (molar ratio 70:25:0.1) grafted with 5% chol-pHPMAlac(43-47), chol-pHPMAlac(82-18), or DSPE-PEG2000 and DC-cholesterol liposomes and AML-12 cells (A), HUVECs (B), HepG2 cells (C) and RAW 264.7 macrophages (D) after 30-minute incubation at standard culture conditions and measurement in a fluorescence plate reader. The degree of association is proportional to the fluorescence intensity of cells. *, $p < 0.01$; **, $p < 0.001$.

2.10. Liposome-induced platelet activation assays

Liposome-induced platelet activation was evaluated for isolated platelets and platelets in whole blood. Blood was drawn from healthy volunteers into citrate-containing Vacutainer tubes (BD Biosciences, Franklin Lakes, NJ). Platelets were isolated according to a previously described method (Korporaal et al., 2007; Yousefi et al., 2014). Blood was centrifuged at 160 × *g* for 15 min at room temperature (RT) to obtain platelet-rich plasma (PRP). Acid citrate dextrose (2.5% w/v trisodium citrate, 1.5% w/v citric acid, and 2% w/v D-glucose) was added to the PRP at a 1:10 ratio (v/v) to lower the pH to 6.5 and chelate liberated calcium ions. PRP was centrifuged at 400 × *g* for 15 min at RT and the platelet pellet was carefully resuspended in HEPES-buffered Tyrode's solution (HT; 145 mM NaCl, 5 mM KCL, 0.5 mM Na_2HPO_4, 1 mM $MgSO_4$, 5.55 mM D-glucose, and 10 mM HEPES, pH = 6.5). Prostacyclin was added at a final concentration of 10 ng/mL to inhibit platelet activation. The platelet suspension was centrifuged at 400 × *g* for 15 min at RT and the pellet was resuspended in HT buffer (pH = 7.3) to a concentration of 2×10^5 platelets/μL. The liposomes (150 μM final phospholipid concentration) were mixed with APC-anti-GP1B (25:1), FITC-anti-fibrinogen (25:1), and PE-anti-P-selectin (12.5:1). TRAP-6, a strong platelet activator (Yousefi et al., 2014), was used as a positive control (208 μM final concentration). The mixtures were incubated at 37 °C for 30 min prior to use, after which 10 μL of isolated platelets or whole blood was added and incubated for 20 min at RT. The incubation was stopped by adding 700 μL fixation buffer (4% w/v formaldehyde and 137 mM NaCl in MilliQ) to the samples. The fluorescence intensity of APC-anti-GP1B, FITC-anti-fibrinogen, and PE-anti-P-selectin was measured by flow cytometry (FACS Canto II, BD Biosciences). Ten thousand events were collected in the platelet gate and data (mean fluorescence intensity, MFI) were analyzed with BD FACSDiva software (BD Biosciences).

2.11. Statistical analysis

Statistical analysis was performed in GraphPad Prism (GraphPad Software, La Jolla, CA). A two-way ANOVA was performed followed by a Bonferroni post-hoc test for statistical analysis of the liposome diameters after incubation in buffer and serum. A one-way ANOVA was performed followed by a Tukey test for statistical analysis of the liposomal association with/uptake by cells.

3. Results and discussion

3.1. Polymer characterization

Previously, a clear correlation between the cloud point of Chol-pHPMAlac polymers and the release temperature of liposomes containing these polymers was found (van Elk et al., 2014). Remarkably, the cloud point of the polymer should be approximately 20–25 °C lower than the temperature used to trigger release from corresponding liposomes. Therefore, in this study Chol-pHPMAlac(43-57) with a low CP (16 °C) was synthesized for validation purposes inasmuch as liposomes grafted with this polymer release their content at mild hyperthermia (van Elk et al., 2014). Chol-pHPMAlac(82-18) with a high CP (42 °C) was prepared as a control because liposomes grafted with this polymer are not expected to release their content at mild hyperthermia.

The physicochemical properties of both polymers are presented in Table 1. The presence of dilactate in the polymer synthesized with HPMA monolactate only (the Chol-pHPMAlac(82-18) formulation) can be explained by dilactate contamination in the starting material, as evidenced by ^{1}H-NMR.

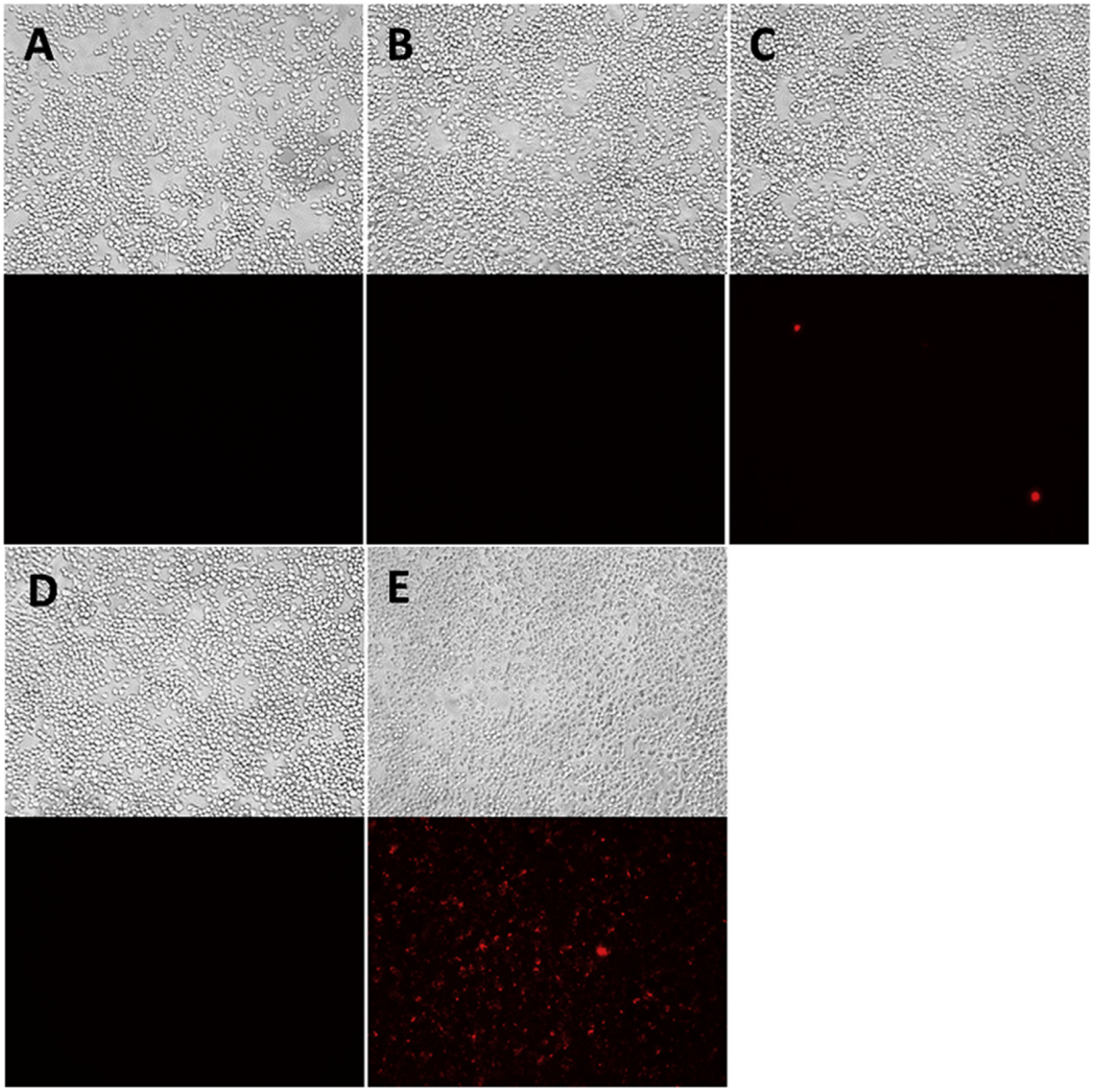

Fig. 4. Phase contrast microscopy images (top row) and corresponding fluorescence microscopy images (bottom row) of RAW 264.7 macrophages incubated for 30 min at standard culture conditions without liposomes (A) or with fluorescently labeled liposomes grafted with 5% chol-pHPMAlac(43-57) (B), chol-pHPMAlac(82-18) (C), or DSPE-PEG2000 (D) and with DC-cholesterol liposomes (E) (150 μM final lipid concentration). The degree of association is proportional to the fluorescence intensity of cells.

3.2. Liposome characterization and doxorubicin release profiles

Liposomes were grafted with chol-pHPMAlac(43-57), chol-pHPMAlac(82-18), or DSPE-PEG2000 (temperature-insensitive polymer) and characterized (Table 2). The size of liposomes grafted with chol-pHPMAlac(82-18) and DSPE-PEG2000 remained unchanged when heated from 10 to 60 °C at a heating rate of 1 °C/min, as evidenced by the unaltered size and PDI. Liposomes grafted with chol-pHPMAlac(43-57) aggregated at 37 °C, given that both size and the PDI increased by approximately 5- and 6-fold, respectively, at this temperature. Chol-pHPMAlac(43-57) starts to dehydrate above its CP at 16 °C, which renders the surface of the liposomes hydrophobic, causing the liposomes to aggregate.

Liposomes grafted with chol-pHPMAlac(43-57) released less than 10% of DOX during 30-minute incubation at 37 °C. Conversely, at 42 °C DOX was released at a rate of 1% per minute, whereas complete DOX release occurred within 10 min at temperatures of ≥47 °C (Fig. 1A), confirming that the release rate was temperature-dependent. In line with expectations, liposomes consisting of DOPE ($T_m = -16$ °C) and EPC grafted with chol-pHPMAlac(82-18) or DSPE-PEG2000 did not release DOX during 30-minute incubation at any of the investigated temperatures (Fig. 1B and C).

3.3. Liposome stability in buffer and serum

The stability of the liposomal formulations was evaluated in PBS and FBS at 37 °C during 1- and 24-hour incubation (i.e., quasi-physiological conditions) using NTA (Fig. 2). No significant change in size was observed for all the liposomal formulations under both conditions, demonstrating that the liposomes retained their integrity in a proteinaceous environment and did not aggregate. PEG is a hydrophilic polymer and forms a steric barrier around the liposomes that increases their colloidal stability and reduces surface adsorption of proteins (Allen et al., 1991; Gabizon et al., 1994; Lasic et al., 1991; Torchilin

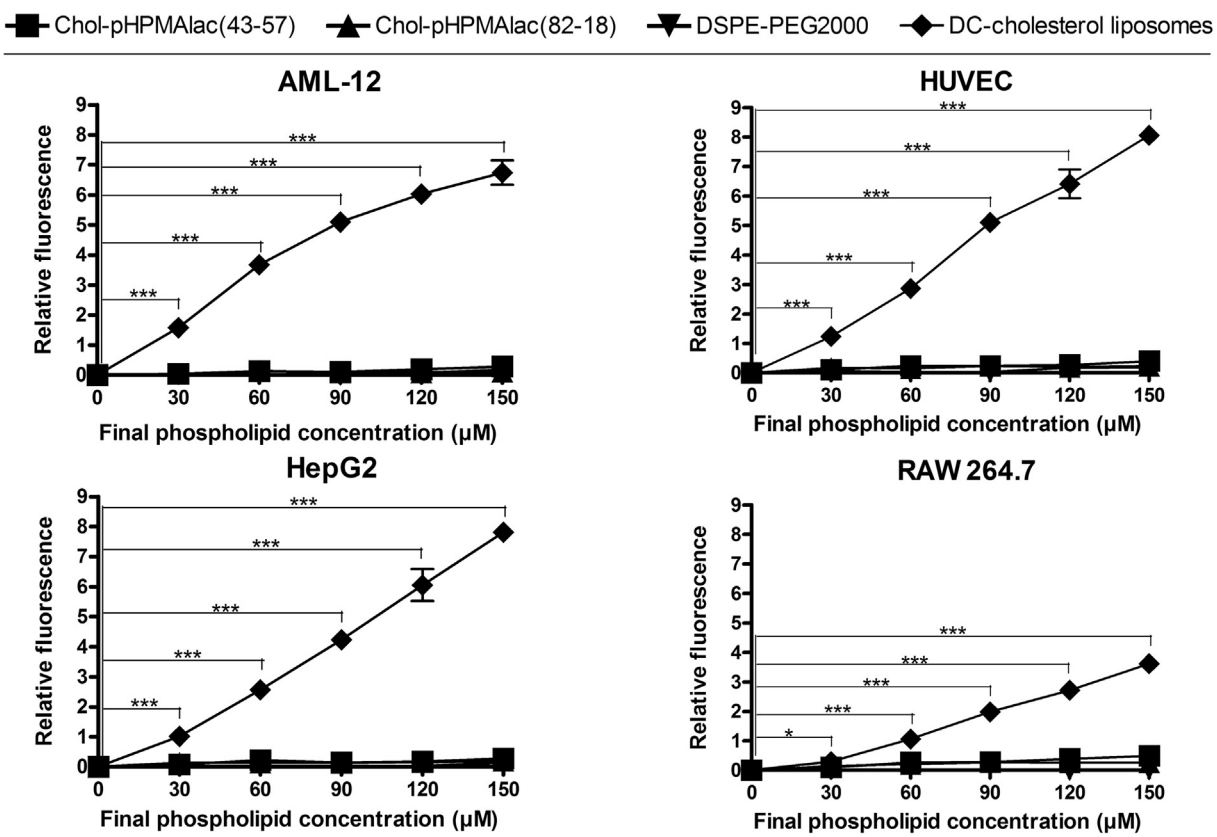

Fig. 5. Association between fluorescently labeled DOPE:EPC:Rho-PE liposomes (70:25:0.1) grafted with 5% chol-pHPMAlac(43-47), chol-pHPMAlac(82-18), or DSPE-PEG2000 and DC-cholesterol liposomes and AML-12 (A), HUVEC (B), HepG2 (C), and RAW 264.7 cells (D) after 24-hour incubation at standard culture conditions and measurement in a fluorescence plate reader. The degree of association is proportional to the fluorescence intensity of cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

et al., 1994). Chol-pHPMAlac is also hydrophilic at temperatures below the CP. Chol-pHPMAlac(82:18) is therefore expected to form a hydrophilic shell around the liposomes similar to PEG.

Interestingly, chol-pHPMAlac(43-57) aggregated at 37 °C (DLS measurements; Table 2), while the size of the liposomes remained unchanged after incubation at 37 °C in buffer and FBS when measured with NTA. The chol-pHPMAlac(43-57) becomes hydrophobic above the CP, which results in aggregation of the liposomes. These (weak) interaction forces are reversible and may be disturbed during agitation of the liposomes during sample preparation for NTA, causing the size of the liposomes to remain unchanged after incubation at 37 °C. Since the sample preparation for DLS measurements is technically different, such agitation effects are absent during the DLS assays, allowing the chol-pHPMAlac-grafted liposomes to aggregate during the measurement. Furthermore, chol-pHPMAlac(43-57)-grafted liposomes exhibited no DOX release at 37 °C, indicating that the lipid bilayer was not perturbed at this temperature despite polymer dehydration and aggregation.

3.4. Association between liposomes and cells

Liposomes can interact with plasma proteins and various types of cells after intravenous administration, which deters selective targeting. A major challenge for tumor-targeted drug delivery systems after intravenous injection is therefore avoiding the uptake by circulating and resident leukocytes and other non-target cells such as endothelial cells that line the vasculature as well as parenchymal cells of detoxification organs such as the liver. Moreover, interaction of liposomes with platelets might result in platelet activation, thrombosis, and vascular occlusion. Accordingly, it is important to get insight into the interaction between chol-pHPMAlac-grafted liposomes with cells and in particularly blood cells that come in contact with the liposomes after intravenous administration.

The association between fluorescently labeled chol-pHPMAlac- and DSPE-PEG2000-grafted liposomes and cultured macrophages (RAW 264.7), HUVECs, and hepatocytes (AML-12) was therefore determined. Furthermore, human hepatocellular carcinoma (HepG2) cells were included in the analysis since the liposomes were designed for the treatment of cancer. Cationic liposomes containing the cationic lipid DC-cholesterol (DPPC:DC-cholesterol:cholesterol:Rho-PE, 70:25:5:0.1, diameter = 156 nm, PDI = 0.24, ζ-potential = 67 ± 9 mV) were used as positive control. Liposomes were added to cells at a maximum phospholipid concentration of 150 μM in these assays since this concentration is clinically relevant (Amantea et al., 1997; Gabizon et al., 2003; Lyass et al., 2000; Mross et al., 2004). When these liposomes are loaded with DOX (2.5 mg/mL) and diluted to a phospholipid concentration of 150 μM, the DOX concentration in these assays will exceed the C_{max} of DOX after intravenous administration of Doxil (Amantea et al., 1997; Gabizon et al., 2003; Lyass et al., 2000; Mross et al., 2004).

Liposomes grafted with chol-pHPMAlac(43-57), chol-pHPMAlac(82-18), or DSPE-PEG2000 did not associate with cells after 30-minute incubation (Fig. 3). The absence of liposome-cell interactions is most likely attributable to the polymers grafted on the surface of the liposomes (forming a hydrophilic shield around the liposomes below the CP of chol-pHPMAlac) and the composition, small size, and near-neutral zeta potential of the liposomes, which are important determinants of uptake (Dan, 2002). Contrastingly, DC-cholesterol liposomes associated with all types of cells in a lipid concentration-dependent manner. It has been reported previously that positively charged nanoparticles bind to the negative surface of cell membranes (He et al., 2010; Yu et al., 2012).

Liposome-cell interactions were also visualized by fluorescence microscopy, which corroborated that there was no association between cells and liposomes grafted with chol-pHPMAlac and DSPE-PEG2000,

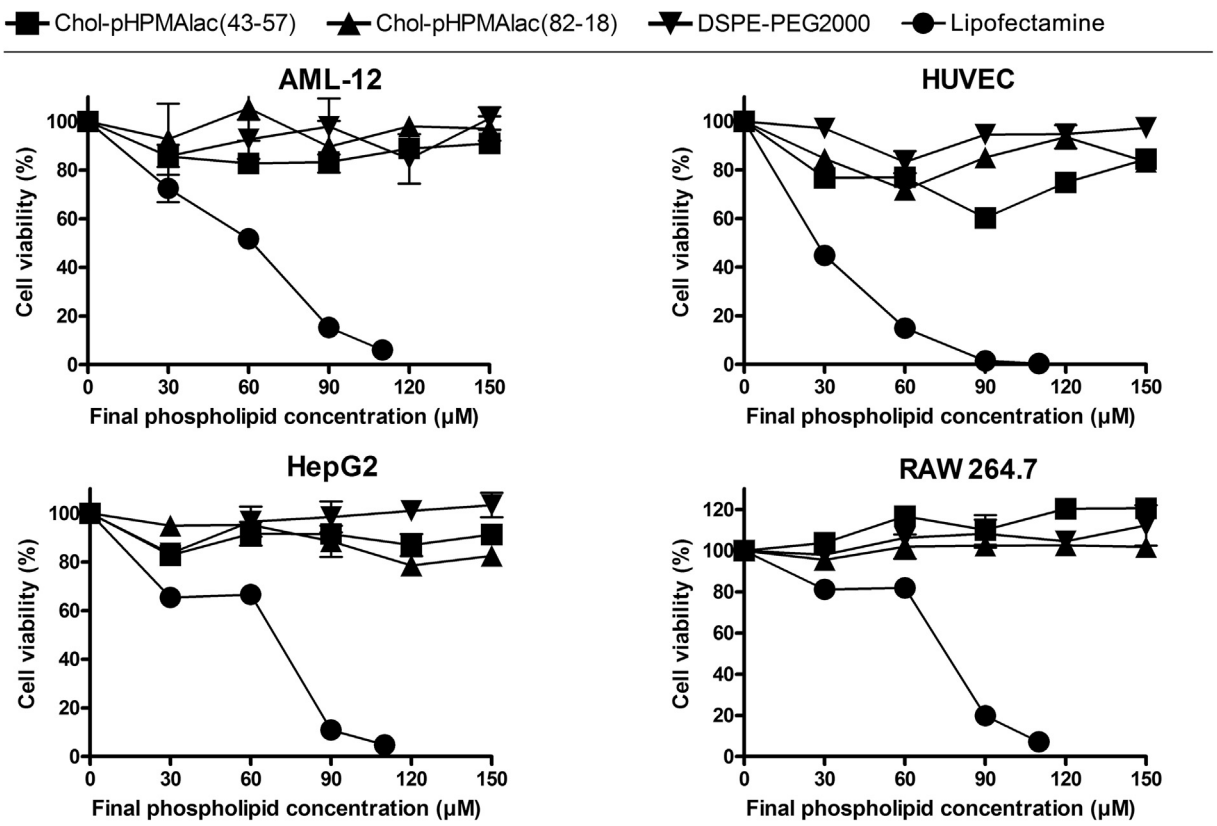

Fig. 6. Viability of AML-12 (A), HUVECs (B), HepG2 (C), and RAW264.7 cells (D) after 24-hour incubation with DOPE:EPC liposomes grafted with 5% chol-pHPMAlac(43-57), chol-pHPMAlac(82-18), or DSPE-PEG2000 and lipofectamine.

while a strong interaction was observed between DC-cholesterol liposomes and all cell types (Fig. 4 and Supplementary Fig. 1).

Finally, liposome-cell interactions were determined after 24-hour incubation with liposomes. This is a more relevant time frame because chol-pHPMAlac-grafted liposomes are expected to undergo relatively slow elimination from the circulation (comparable to PEGylated liposomes) and reside in the tumor for an extended period of time (Ngoune et al., 2016) despite lymphatic drainage (Swartz and Lund, 2012). Liposomes grafted with chol-pHPMAlac(43-57), chol-pHPMAlac(82-18), or DSPE-PEG2000 exhibited only a marginal increase in cell association when incubated for 24 h compared to 30-minute incubation (Fig. 5 and Supplementary Fig. 2). In contrast, DC-cholesterol liposomes exhibited a profound increase in cell association following 24-hour incubation versus 30-minute incubation (Fig. 3). Taken altogether, liposomes grafted with chol-pHPMAlac showed no notable association with macrophages, endothelial cells, hepatocytes, and hepatocellular carcinoma cells. These liposomes are therefore not likely to be taken up directly after intravenous administration, which increases the probability that the liposomes reach the target site (tumor tissue) after systemic administration, where the chemotherapeutic payload can be released locally upon the induction of mild hyperthermia (see Fig. 6).

3.5. Cytotoxicity of liposomal formulations

Chol-pHPMAlac-grafted liposomes did not associated with or were taken up by various cell types, therefore these liposomes most likely do not induce cytotoxicity. To confirm this hypothesis, the cytotoxicity of the liposomal formulations was determined by incubating cells with the different liposomal formulations and assaying viability after 24 h with the MTS assay. Lipofectamine 2000 was used as positive control since this liposomal formulation is known to be cytotoxic (Jain, Kumar et al., 2013). The MTS assay revealed that liposomes grafted with chol-pHPMAlac or DSPE-PEG2000 had a minimal-to-negligible effect on cell viability up to a lipid concentration of 150 μM. The cell viability decreased to < 20% when lipofectamine was incubated with the cells at concentrations of ≥90 μM. Since the phospholipid concentration will not exceed 150 μM after infusion into patients (based on the DOX concentration loaded into these liposomes and the C_{max} achieved in patients after administration of Doxil (Amantea et al., 1997; Gabizon et al., 2003; Lyass et al., 2000; Mross et al., 2004)) it is not expected that these liposomes will induce toxicity after intravenous administration.

3.6. Platelet activation

Platelets play an important role in hemostasis, wound healing, immunity, and tumor biology (Elaskalani et al., 2017; Heger et al., 2014). Following activation, platelets release an armament of bioactive molecules and particles that affect platelets and multiple cell types, including cancer cells (Goubran et al., 2014), through which they modulate biological processes. Activated platelets release fibrinogen from the α-granules. The released fibrinogen binds to integrin αIIbβ3 on the activated platelets and forms crosslinks between adjacent platelets, leading to platelet aggregation and fortification of the platelet plug (Broos et al., 2012; Broos et al., 2011; Nieswandt et al., 2009). In addition, P-selectin (CD62P) translocates to the membrane outer surface upon activation and stabilizes the interplatelet fibrinogen bridges. Moreover, P-selectin mediates the tethering and accumulation of platelets at the site of injury (Armstrong and Peter, 2012; Merten and Thiagarajan, 2000). The density of fibrinogen and P-selectin on the surface of the platelets is a good indicator for platelet activation. Accordingly, platelet activation was assessed in the context of our liposomal formulations to ensure that the liposomes do not induce platelet activation and corollary thromboembolic complications and tumor-promoting signaling following systemic administration. Thrombin receptor activating peptide (TRAP-6) was used as positive control since

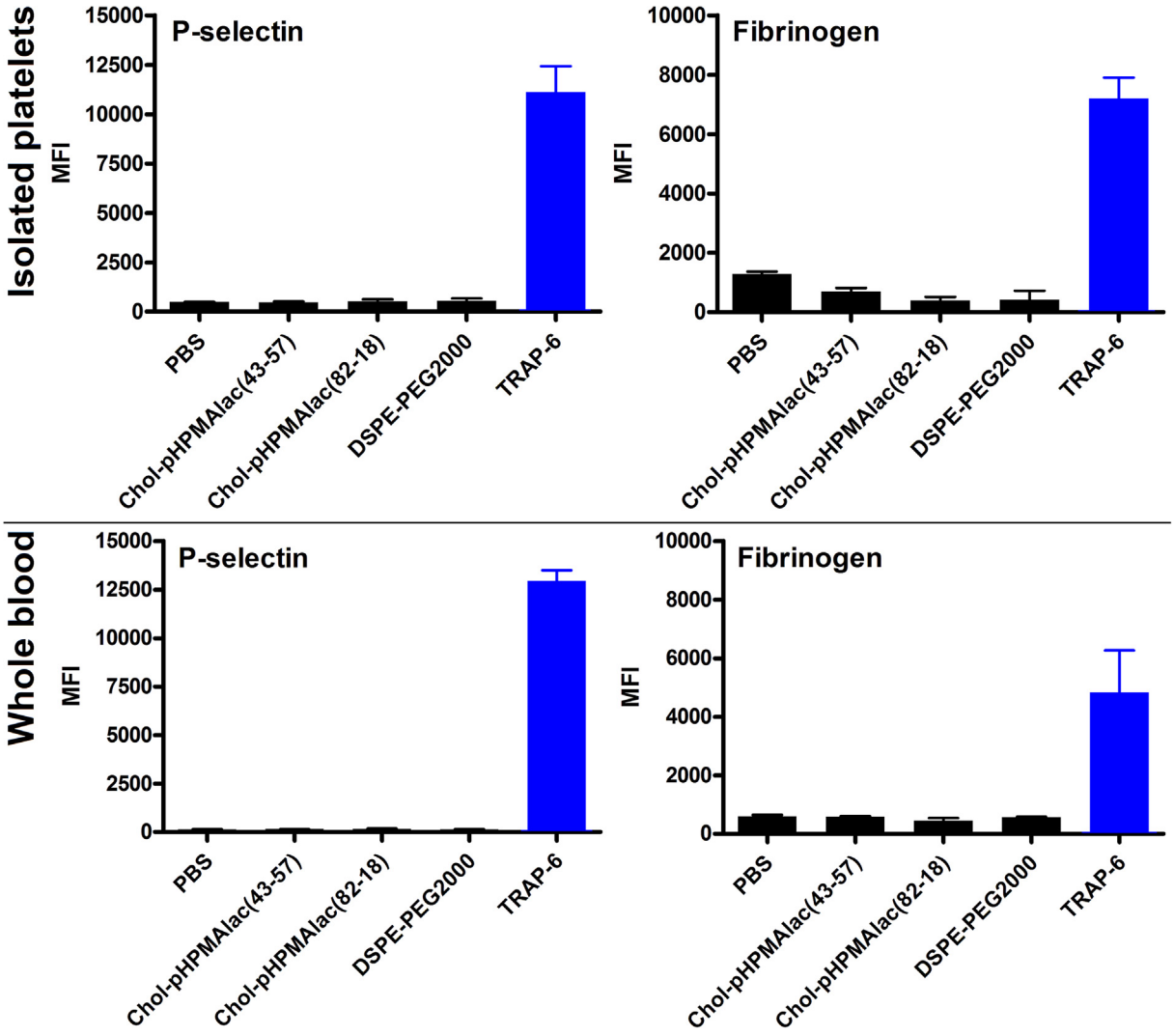

Fig. 7. Platelet activation by liposomes grafted with chol-pHPMAlac(43-57), chol-pHPMAlac(82-18), or DSPE-PEG2000 in isolated platelets (A-B) and whole blood (C-D). The mean fluorescence intensity (MFI) is proportional to the degree of P-selectin expression (A + C) and fibrinogen binding (B + D). TRAP-6 was used as positive control (blue bar) and represents complete platelet activation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

TRAP-6 activates αIIbβ3 receptors and induces P-selectin expression. Liposomes grafted with chol-pHPMAlac or DSPE-PEG2000 did not induce the expression of P-selectin and the binding of fibrinogen on isolated platelets after 20-minute incubation (Fig. 7A-B).

Activation of platelets was also determined in whole blood, which better mimics the in vivo situation compared to isolated platelets and is therefore more clinically relevant. The extent of fibrinogen binding and P-selectin activation remained unaltered following incubation of whole blood with chol-pHPMAlac- and DSPE-PEG2000-grafted liposomes, confirming that the liposomes do not activate platelets (Fig. 7C-D) and therefore pose no danger in the context of thrombosis as well as paracrine immunomodulation and tumor biology.

4. Conclusions

The objectives of this study were to get insight into (1) the stability of the chol-pHPMAlac-TSLs in quasi-biological circumstances and (2) the interaction between chol-pHPMAlac-grafted liposomes with cells with which the liposomes may interact after intravenous administration. The data demonstrate that the chol-pHPMAlac TSLs are stable and heat-activatable in a biological milieu, non-associative and therefore non-toxic to cancer and healthy cells, and safe for systemic administration in terms of being non-antagonistic to platelets. Overall, these results suggest that chol-pHPMAlacTSLs are not expected to be rapidly cleared from the circulation and most likely do not induce detrimental biological processes after injection. Consequently, liposomes grafted with chol-pHPMAlac constitute a promising carrier system for intratumoral chemotherapeutic delivery and local temperature-triggered release. In vivo follow-up studies are therefore warranted to devlop the formulation further for systemic and targeted cancer therapies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijpharm.2018.08.027.

References

Allen, T.M., Hansen, C., Martin, F., Redemann, C., Yau-Young, A., 1991. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. Biochim. Biophys. Acta 1066, 29–36.

Amantea, M.A., Forrest, A., Northfelt, D.W., Mamelok, R., 1997. Population pharmacokinetics and pharmacodynamics of pegylated-liposomal doxorubicin in patients with AIDS-related Kaposi's sarcoma. Clin. Pharmacol. Ther. 61, 301–311.

Armstrong, P.C., Peter, K., 2012. GPIIb/IIIa inhibitors: from bench to bedside and back to bench again. Thromb. Haemost. 107, 808–814.

Bandak, S., Goren, D., Horowitz, A., Tzemach, D., Gabizon, A., 1999. Pharmacological studies of cisplatin encapsulated in long-circulating liposomes in mouse tumor models. Anticancer Drugs 10, 911–920.

Broekgaarden, M., de Kroon, A.I., van Gulik, T.M., Heger, M., 2014. Development and in vitro proof-of-concept of interstitially targeted zinc-phthalocyanine liposomes for photodynamic therapy. Curr. Med. Chem. 21, 377–391.

Broos, K., Feys, H.B., De Meyer, S.F., Vanhoorelbeke, K., Deckmyn, H., 2011. Platelets at work in primary hemostasis. Blood Rev. 25, 155–167.

Broos, K., De Meyer, S.F., Feys, H.B., Vanhoorelbeke, K., Deckmyn, H., 2012. Blood platelet biochemistry. Thromb. Res. 129, 245–249.

Dan, N., 2002. Effect of liposome charge and PEG polymer layer thickness on cell-liposome electrostatic interactions. Biochim. Biophys. Acta 1564, 343–348.

Elaskalani, O., Berndt, M.C., Falasca, M., Metharom, P., 2017. Targeting platelets for the treatment of cancer. Cancers (Basel.) 9, 94.

Gaber, M.H., Wu, N.Z., Hong, K., Huang, S.K., Dewhirst, M.W., Papahadjopoulos, D., 1996. Thermosensitive liposomes: extravasation and release of contents in tumor microvascular networks. Int. J. Radiat. Oncol. Biol. Phys. 36, 1177–1187.

Gabizon, A., Catane, R., Uziely, B., Kaufman, B., Safra, T., Cohen, R., Martin, F., Huang, A., Barenholz, Y., 1994. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. Cancer Res. 54, 987–992.

Gabizon, A., Shmeeda, H., Barenholz, Y., 2003. Pharmacokinetics of pegylated liposomal Doxorubicin: review of animal and human studies. Clin. Pharmacokinet. 42, 419–436.

Goubran, H.A., Kotb, R.R., Stakiw, J., Emara, M.E., Burnouf, T., 2014. Regulation of tumor growth and metastasis: the role of tumor microenvironment. Cancer Growth Metastasis 7, 9–18.

Hahn, G.M., Braun, J., Har-Kedar, I., 1975. Thermochemotherapy: synergism between hyperthermia (42–43 degrees) and adriamycin (of bleomycin) in mammalian cell inactivation. Proc. Natl. Acad. Sci U.S.A. 72, 937–940.

He, C., Hu, Y., Yin, L., Tang, C., Yin, C., 2010. Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. Biomaterials 31, 3657–3666.

Heger, M., Salles, I.I., de Kroon, A.I., Deckmyn, H., 2009a. Platelets and PEGylated lecithin liposomes: when stealth is allegedly picked up on the radar (and eaten). Microvasc. Res. 78, 1–3.

Heger, M., Salles, I.I., van Vuure, W., Hamelers, I.H., de Kroon, A.I., Deckmyn, H., Beek, J.F., 2009b. On the interaction of fluorophore-encapsulating PEGylated lecithin liposomes with hamster and human platelets. Microvasc. Res. 78, 57–66.

Heger, M., van Golen, R.F., Broekgaarden, M., van den Bos, R.R., Neumann, H.A., van Gulik, T.M., van Gemert, M.J., 2014. Endovascular laser-tissue interactions and biological responses in relation to endovenous laser therapy. Lasers Med. Sci. 29, 405–422.

Herman, T.S., 1983. Temperature dependence of adriamycin, cis-diamminedichloroplatinum, bleomycin, and 1,3-bis(2-chloroethyl)-1-nitrosourea cytotoxicity in vitro. Cancer Res. 43, 517–520.

Jain, S., Kumar, S., Agrawal, A.K., Thanki, K., Banerjee, U.C., 2013. Enhanced transfection efficiency and reduced cytotoxicity of novel lipid-polymer hybrid nanoplexes. Mol. Pharm. 10, 2416–2425.

Kong, G., Anyarambhatla, G., Petros, W.P., Braun, R.D., Colvin, O.M., Needham, D., Dewhirst, M.W., 2000. Efficacy of liposomes and hyperthermia in a human tumor xenograft model: importance of triggered drug release. Cancer Res. 60, 6950–6957.

Kong, G., Braun, R.D., Dewhirst, M.W., 2001. Characterization of the effect of hyperthermia on nanoparticle extravasation from tumor vasculature. Cancer Res. 61, 3027–3032.

Korporaal, S.J., Van, E.M., Adelmeijer, J., Ijsseldijk, M., Out, R., Lisman, T., Lenting, P.J., Van Berkel, T.J., Akkerman, J.W., 2007. Platelet activation by oxidized low density lipoprotein is mediated by CD36 and scavenger receptor-A. Arterioscler. Thromb. Vasc. Biol. 27, 2476–2483.

Landon, C.D., Park, J.Y., Needham, D., Dewhirst, M.W., 2011. Nanoscale drug delivery and hyperthermia: the materials design and preclinical and clinical testing of low temperature-sensitive liposomes used in combination with mild hyperthermia in the treatment of local cancer. Open Nanomed. J. 3, 38–64.

Lasic, D.D., Martin, F.J., Gabizon, A., Huang, S.K., Papahadjopoulos, D., 1991. Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. Biochim. Biophys. Acta 1070, 187–192.

Limmer, S., Hahn, J., Schmidt, R., Wachholz, K., Zengerle, A., Lechner, K., Eibl, H., Issels, R.D., Hossann, M., Lindner, L.H., 2014. Gemcitabine treatment of rat soft tissue sarcoma with phosphatidyldiglycerol-based thermosensitive liposomes. Pharm. Res. 31, 2276–2286.

Liu, Y., Cho, C.W., Yan, X., Henthorn, T.K., Lillehei, K.O., Cobb, W.N., Ng, K.Y., 2001. Ultrasound-Induced hyperthermia increases cellular uptake and cytotoxicity of P-glycoprotein substrates in multi-drug resistant cells. Pharm. Res. 18, 1255–1261.

Lyass, O., Uziely, B., Ben-Yosef, R., Tzemach, D., Heshing, N.I., Lotem, M., Brufman, G., Gabizon, A., 2000. Correlation of toxicity with pharmacokinetics of pegylated liposomal doxorubicin (Doxil) in metastatic breast carcinoma. Cancer 89, 1037–1047.

Manzoor, A.A., Lindner, L.H., Landon, C.D., Park, J.Y., Simnick, A.J., Dreher, M.R., Das, S., Hanna, G., Park, W., Chilkoti, A., Koning, G.A., ten Hagen, T.L., Needham, D., Dewhirst, M.W., 2012. Overcoming limitations in nanoparticle drug delivery: triggered, intravascular release to improve drug penetration into tumors. Cancer Res. 72, 5566–5575.

Matteucci, M.L., Anyarambhatla, G., Rosner, G., Azuma, C., Fisher, P.E., Dewhirst, M.W., Needham, D., Thrall, D.E., 2000. Hyperthermia increases accumulation of technetium-99m-labeled liposomes in feline sarcomas. Clin. Cancer Res. 6, 3748–3755.

Merten, M., Thiagarajan, P., 2000. P-selectin expression on platelets determines size and stability of platelet aggregates. Circulation 102, 1931–1936.

Mross, K., Niemann, B., Massing, U., Drevs, J., Unger, C., Bhamra, R., Swenson, C.E., 2004. Pharmacokinetics of liposomal doxorubicin (TLC-D99; Myocet) in patients with solid tumors: an open-label, single-dose study. Cancer Chemother. Pharmacol. 54, 514–524.

Needham, D., Anyarambhatla, G., Kong, G., Dewhirst, M.W., 2000. A new temperature-sensitive liposome for use with mild hyperthermia: characterization and testing in a human tumor xenograft model. Cancer Res. 60, 1197–1201.

Neradovic, D., van Steenbergen, M.J., Vansteelant, L., Meijer, Y.J., van Nostrum, C.F., Hennink, W.E., 2003. Degradation mechanism and kinetics of thermosensitive polyacrylamides containing lactic acid side chains. Macromolecules 36, 7491–7498.

Ngoune, R., Peters, A., von Elverfeldt, D., Winkler, K., Putz, G., 2016. Accumulating nanoparticles by EPR: a route of no return. J. Control. Release 238, 58–70.

Nieswandt, B., Varga-Szabo, D., Elvers, M., 2009. Integrins in platelet activation. J. Thromb. Haemost. 7 (Suppl. 1), 206–209.

Paasonen, L., Romberg, B., Storm, G., Yliperttula, M., Urtti, A., Hennink, W.E., 2007. Temperature-sensitive poly(N-(2-hydroxypropyl)methacrylamide mono/dilactate)-coated liposomes for triggered contents release. Bioconjug. Chem. 18, 2131–2136.

Swartz, M.A., Lund, A.W., 2012. Lymphatic and interstitial flow in the tumour microenvironment: linking mechanobiology with immunity. Nat. Rev. Cancer 12, 210–219.

Tagami, T., May, J.P., Ernsting, M.J., Li, S.D., 2012. A thermosensitive liposome prepared with a Cu(2)(+) gradient demonstrates improved pharmacokinetics, drug delivery and antitumor efficacy. J. Control. Release 161, 142–149.

Torchilin, V.P., Omelyanenko, V.G., Papisov, M.I., Bogdanov Jr., A.A., Trubetskoy, V.S., Herron, J.N., Gentry, C.A., 1994. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. Biochim. Biophys. Acta 1195, 11–20.

van Elk, M., Deckers, R., Oerlemans, C., Shi, Y., Storm, G., Vermonden, T., Hennink, W.E., 2014. Triggered release of doxorubicin from temperature-sensitive poly(N-(2-hydroxypropyl)-methacrylamide mono/dilactate) grafted liposomes. Biomacromolecules 15, 1002–1009.

van Raath, M.I., Weijer, R., Nguyen, G.H., Choi, B., de Kroon, A.I., Heger, M., 2016. Tranexamic acid-encapsulating thermosensitive liposomes for site-specific pharmaco-laser therapy of port wine stains. J. Biomed. Nanotechnol. 12, 1617–1640.

Yarmolenko, P.S., Zhao, Y., Landon, C., Spasojevic, I., Yuan, F., Needham, D., Viglianti, B.L., Dewhirst, M.W., 2010. Comparative effects of thermosensitive doxorubicin-containing liposomes and hyperthermia in human and murine tumours. Int. J. Hyperthermia 26, 485–498.

Yousefi, A., Lauwers, M., Nemes, R., van Holten, T., Babae, N., Roest, M., Storm, G., Schiffelers, R., Mastrobattista, E., 2014. Hemocompatibility assessment of two siRNA nanocarrier formulations. Pharm. Res. 31, 3127–3135.

Yu, B., Zhang, Y., Zheng, W., Fan, C., Chen, T., 2012. Positive surface charge enhances selective cellular uptake and anticancer efficacy of selenium nanoparticles. Inorg. Chem. 51, 8956–8963.