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Proteins and cholesterol lipid vesicles are mediators of drug release from thermosensitive liposomes

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ABSTRACT

Thermosensitive liposomes (TSL) are a promising tool for triggered drug delivery in combination with local hyperthermia. Objective of this study was to investigate the influence of serum on TSL in more detail and to identify serum components which are responsible for increasing drug release. Four different formulations were investigated: DPPC/DSPC/1,2-dipalmitoyl-*sn*-glycero-3-phosphodiglycerol ($DPPG_2$) 50/20/30 (mol/mol) ($DPPG_2$-TSL); DPPC/DSPC/$DPPG_2$/DSPE-PEG2000 50/15/30/5 (mol/mol) ($DPPG_2$/PEG-TSL), DPPC/P-Lyso-PC/DSPE-PEG2000 90/10/4 (mol/mol) (PEG/Lyso-TSL), and DPPC/DSPC/DSPE-PEG2000 80/15/5 (mol/mol) (PEG-TSL). $DPPG_2$-TSL was the only formulation which was unaffected by osmotic stress. All formulations tested were influenced by serum components but the susceptibility was depended on the lipid composition of the vesicle. Presence of albumin (HSA) or cholesterol-containing lipid vesicles (DPPC/Chol-LLV) increased the membrane permeability for all tested formulations at temperatures around and above T_m in a concentration based manner. PEGylation was not able to prevent the observed effect. PEG-TSL and PEG/Lyso-TSL were more susceptible to DPPC/Chol-LLV than $DPPG_2$-containing TSL. In contrast, immunoglobulin type G (IgG) affected only anionic formulations. The membrane of $DPPG_2$-TSL and $DPPG_2$/PEG-TSL was more susceptible toward IgG as compared to HSA. $DPPG_2$-TSL and PEG/Lyso-TSL were differentially influenced by fetal calf serum (FCS). As $DPPG_2$-TSL was stabilized by pre-incubation with FCS at 37 °C, this was the opposite for PEG/Lyso-TSL which were destabilized under these conditions. Individual serum components were unable to mimic the complex situation in full serum. Hence, the use of plasma or serum is still inevitable to investigate stability and release properties of novel TSL formulations until all serum components have been identified that alter TSL integrity.

1. Introduction

Thermosensitive liposomes (TSL) are a promising tool for targeted and triggered drug delivery to solid tumors in combination with regional hyperthermia or high-intensity focused ultrasound [1–9]. The drug is safely encapsulated inside the TSL at body temperature when circulating in the bloodstream. After reaching the heated tumor area the drug is released due to a permeability change of the TSL bilayer membrane. Release generally occurs by passive transfer across the membrane according to a concentration gradient. The permeability across the membrane depends on the phase state of the bilayer forming lipids and the molecular structure of the drug [1,5]. At body temperature the membrane is in the solid gel phase state, characterized by low permeability for hydrophilic compounds. Above its phase transition temperature (T_m), the membrane changes to the liquid disordered phase state, with markedly increased permeability. Around T_m the permeability is largest due the coexistence of membrane areas in both phases forming boundaries with packing defects [2,10]. In addition, for lysolipid-containing

Abbreviations: Chol, cholesterol; CF, carboxyfluorescein; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPC-LLV, large multilamellar DPPC lipid vesicle; DPPC/Chol-LLV, large multilamellar DPPC/Chol 55/45 (mol/mol) lipid vesicle; DPPC/Chol-SLV, small unilamellar DPPC/Chol 55/45 (mol/mol) lipid vesicle; $DPPG_2$, 1,2-dipalmitoyl-*sn*-glycero-3-phosphodiglycerol; $DPPG_2$-TSL, liposomes composed of DPPC/DSPC/$DPPG_2$ 50/20/30 (mol/mol); $DPPG_2$/PEG-TSL, liposomes composed of DPPC/DSPC/$DPPG_2$/DSPE-PEG2000 50/15/30/5 (mol/mol); DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPE-PEG2000, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-methoxy(PEG)-2000; FCS, fetal calf serum; IgG, human immunoglobulin G; HSA, human serum albumin; i.v., intravenous; P-Lyso-PC, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; PEG-TSL, liposomes composed of DPPC/DSPC/DSPE-PEG2000 80/15/5 (mol/mol); PEG/Lyso-TSL, liposomes composed of DPPC/P-Lyso-PC/DSPE-PEG2000 90/10/4 (mol/mol); T_m, solid gel to liquid disordered phase transition temperature; TSL, thermosensitive liposomes.

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formulations (e.g. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (P-Lyso-PC)/1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-methoxy(PEG)-2000 (DSPE-PEG2000) 90/10/4 (mol/mol) (PEG/Lyso-TSL)) the formation of lysolipid stabilized pores was described as mechanism for an ultrafast drug release [2,11]. T_m is adjustable by the phospholipid composition of the carrier [4]. But also changes in vesicle size alter the release profile of TSL without affecting T_m [5,12].

It is well known that presence of serum is an essential prerequisite to increase the drug release rate from TSL [4,5,13,14]. Since TSL are mainly located in the bloodstream after application it is crucial to identify the impact of individual serum components on vesicle integrity. This was intensively studied for liposomes over the last decades, but less is known about it for current TSL formulations. Serum components have to interact with a stressful environment due to the different attractive and repulsive interactions between the membrane forming phospholipids [15]. The interaction is an oxygen-dependent, exothermic process [16]. Proteins adsorb to liposomes [17] and subsequently affect the integrity of the membrane bilayer by partially penetrating into it [18–20]. Albumin (HSA) and immunoglobulin type G (IgG) are the major protein components in human blood (up to 88% of total protein). Both proteins interact with liposomes and increase drug leakage [14,20–22]. But also proteins from the complement system gained reasonable clinical interest, since they might yield to serious hypersensitivity reactions in patients upon intravenous (i.v.) application of liposomes [23]. Human blood contains between 4 and 10 g/l of lipids which are mainly associated to cells and lipoproteins due to their low solubility in aqueous systems. Phospholipids are mainly exchanged between vesicles and lipoproteins by transfer proteins [24–26]. In contrast, cholesterol (Chol) [27] and micelle-forming lysolipids [28] freely exchange between vesicles. Lipid exchange or loss disturbs the bilayer packing and yields to an increased permeability of the membrane bilayer [24,28,29].

Our objective in the present study was to investigate the influence of serum on TSL in more detail and to identify serum components responsible for altering the stability and drug release of four different formulations: 1) PEG/Lyso-TSL recently entered human clinical trials with a similar formulation [2]. 2) DPPC/1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)/1,2-dipalmitoyl-*sn*-glycero-3-phosphodiglycerol ($DPPG_2$) 50/20/30 (mol/mol) TSL ($DPPG_2$-TSL) exhibit a linear pharmacokinetic profile with increased circulation half-life without PEGylation [3]. In vitro they exhibit an ultra-fast doxorubicin (DOX) release at 42 °C and a superior stability in serum at 37 °C over PEG/Lyso-TSL [4]. 3) $DPPG_2$/PEG-TSL: DPPC/DSPC/$DPPG_2$/DSPE-PEG2000 50/15/30/5 (mol/mol) have been chosen to shield the negative ζ-potential of the anionic phospholipid $DPPG_2$ by PEGylation. 4) PEG-TSL: DPPC/DSPC/DSPE-PEG2000 80/15/5 (mol/mol) is a candidate for passive accumulation in the tumor tissue prior to heat triggered drug release [4,6].

2. Materials and methods

2.1. Chemicals

The phospholipid $DPPG_2$ was synthesized as described before [30]. DSPE-PEG2000 was obtained from Avanti Polar Lipids (Alabaster, Alabama, USA). DPPC, DSPC and P-Lyso-PC were purchased from Genzyme Pharmaceuticals (Liestal, Switzerland). CF free acid (Fluka, Buchs, Switzerland) was transformed to its sodium salt with sodium hydroxide and additionally purified by crystallization. HSA (Albunorm® 200 g/l) was from Octapharma GmbH (Langenfeld, Germany). IgG (Intradect®, 50 g/l) was purchased from Biotest Pharma GmbH (Dreieich, Germany). Fetal calf serum (FCS) was from Biochrom AG (Berlin, Germany). Pooled human plasma was a gift of the blood bank of the University hospital of Munich. Plasma from different species was from Sera Laboratories International Ltd (Haywards Heath, UK). All other chemicals were either from Carl Roth GmbH (Karlsruhe, Germany) or Sigma Aldrich GmbH (Munich, Germany).

2.2. Preparation and characterization of TSL

TSL were prepared and characterized as described in detail elsewhere [4,5]. In brief, TSL were prepared by the lipid film hydration and extrusion method. CF was passively encapsulated by hydration of the lipid film with 100 mM CF pH 7.2. DOX was actively loaded to TSL encapsulating 300 mM citrate pH 4 with an extraliposomal pH of 7.8. T_m was measured with differential scanning calorimetry (DSC). The hydrodynamic diameter and ζ-potential were determined by dynamic light scattering (DLS). Phospholipid concentration was determined by phosphate analysis. Phospholipid composition was quantitatively measured with thin layer chromatography (TLC). CF and DOX were quantified with fluorescence spectroscopy.

2.3. Temperature dependent content release

CF and DOX release were measured as described previously [4]. In brief, each TSL sample was diluted 1/10 (vol/vol) in the desired buffer, physiological saline with supplemented serum components or FCS at room temperature. Twenty μl of the dilution were incubated in a pre-heated thermoshaker (Eppendorf, Hamburg, Germany), so the sample reached the desired incubation temperature within a few seconds. Please refer to the results section for the particular conditions. Subsequently, each sample was diluted with ice cold buffer to a final volume of 1 ml to stop the experiment. As buffer Tris/NaCl 0.9% buffer [10 mM (pH 8.0)] (CF) or 20 mM HEPES, 150 mM NaCl, pH 7.4 (DOX) have been used to adjust the desired pH for the fluorescence intensity measurements.

2.4. Pre-incubation of TSL

TSL samples were diluted 1/10 (vol/vol) in FCS, transferred to a pre-heated thermoshaker (37 °C) and incubated for 1 h under shaking. Subsequently, 20 μl was transferred to a fresh tube and immediately incubated in a second thermoshaker at the desired temperature to measure the temperature dependent CF release during 5 min as described above.

2.5. Fluorescence spectroscopy

The fluorescence intensity was measured in a Cary Eclipse fluorescence spectrometer (Varian Inc., Palo Alto, CA, USA) at room temperature (CF: excitation wavelength 493 nm/emission wavelength 513 nm; DOX: 470 nm/555 nm). Percentage release was calculated as: $\text{Release}[\%] = (I(t) - I_0)/(I_\infty - I_0) * 100$. $I(t)$ is the fluorescence intensity after incubation for the time period t and I_0 is the fluorescence baseline. The fluorescence intensity for 100% release (I_∞) was obtained after treatment of a sample with 10% Triton X-100 in water (15 min/45 °C).

2.6. Statistical analysis

The data are expressed as the mean ± standard deviation of at least three independent experiments.

3. Results

3.1. Species dependent effect of plasma on doxorubicin release from $DPPG_2$-TSL

Blood is a complex environment for TSL with a high diversity of components showing marked differences between species, genders and individuals (Supplementary Table 1). $DPPG_2$-TSL encapsulating the chemotherapeutic agent DOX was prepared to investigate the influence of plasma of distinct species on the temperature dependent DOX release of a clinically relevant formulation (Fig. 1).