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Research paper

Impact of plasma protein binding on cargo release by thermosensitive liposomes probed by fluorescence correlation spectroscopy

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ABSTRACT

Thermosensitive liposomes (TSLs) whose phase-transition temperature (T_m) lies slightly above body temperature are ideal candidates for controlled drug release via local hyperthermia. Recent studies, however, have revealed disruptive shifts in the release temperature T_r in mouse plasma, which are attributed to undefined interactions with blood proteins. Here, we study the effects of four major plasma proteins – serum albumin (SA), transferrin (Tf), apolipoprotein A1 (ApoA1) and fibrinogen (Fib) – on the temperature-dependent release of fluorescein di-β-D-galactopyranoside (FDG) from TSLs. The amount of fluorescein released was quantified by fluorescence correlation spectroscopy (FCS) after hydrolysis of FDG with β-galactosidase (β-Gal). This approach is more sensitive and thus superior to previous release assays, as it is impervious to the confounding effects of Triton on conventional fluorescence measurements. The assay determines the molar release ratio, i.e. the number of molecules released per liposome. We show that shifts in the T_r of release do not reflect protein affinities for the liposomes derived from adsorption isotherms. We confirm a remarkable shift in induced release towards lower temperatures in the presence of mouse plasma. In contrast, exposure to rat or human plasma, or fetal bovine serum (FBS), has no effect on the release profile.

1. Introduction

In the 1970s, Gregory Gregoriadis first proposed the use of liposomes for drug delivery [1–3]. Liposomes have many obvious advantages in this context: they are biocompatible, biodegradable and show little or no antigenic or allergenic activity. These features largely explain why the majority of FDA-approved and therapeutically applied nanoparticle-based delivery vehicles falls into the category of liposomal or lipid-based formulations, such as Doxil, DaunoXome or Marqibo [4–8]. A potential limitation of these products is the absence of a controllable drug release mechanism, which would enable localized therapy and thus reduce side-effects. Therefore, the next step in the development of lipid-based delivery platforms was the implementation of stimulus-responsive drug delivery. Thermosensitive liposomes (TSLs) are the most promising triggered systems, with one formulation based on lyso-lipids (LTSL) [9,10] currently tested in various clinical studies up to phase 3 in humans (Thermodox) [11,12]. TSLs allow for temperature-controlled drug release in response to local hyperthermia or focused high-intensity ultrasound [13–16].

TSLs are composed of a lipid mixture that is designed to have a melting phase-transition temperature (T_m) a few degrees above a chosen target temperature. At this point, the structure of the lipid bilayer changes from a solid-gel phase to a liquid-crystalline phase. This change increases the permeability of the membrane for the

Abbreviations: ApoA1, apolipoprotein A1; β-Gal, β-galactosidase; CF, carboxyfluorescein; DSC, differential scanning calorimetry; DLS, dynamic light scattering; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; $DPPG_2$, 1,2-dipalmitoyl-*sn*-glycero-3-phosphodiglycerol; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; FBS, fetal bovine serum; F(C)S, fluorescence (correlation) spectroscopy; FDG, fluorescein di-β-D-galactopyranoside; Fib, fibrinogen; LTSL, low temperature sensitive liposomes; Lyso-PC, 1-acyl-*sn*-glycero-3-phosphocholine; SA, serum albumin; T_m, temperature of the transition from the solid gel to the liquid disordered phase; TSL, thermosensitive liposome; Tf, transferrin; Tr, release temperature.

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encapsulated drug at temperatures in the vicinity of T_m, leading to the release of the drug by passive transfer along a concentration gradient [13]. In the present study, we use TSLs composed of DPPC/DSPC/$DPPG_2$ (50/20/30 mol/mol) ($DPPG_2$-TSL) as proposed by Lindner et al. [17]. The phase transition takes place at $T_m \sim 42$ °C, i.e., above mammalian body temperature [18,19]. This formulation proved to be more stable in serum than the more generally used LTSL [20]. Presence of serum components at 37 °C stabilized $DPPG_2$-TSL over time, whereas for LTSL the opposite was found. LTSL were more susceptible towards destabilizing effects by cholesterol-containing vesicles, whereas presence of proteins stronger affected $DPPG_2$-TSL regarding to both stabilizing (e.g. albumin) and destabilizing effects (e.g. immunoglobulin type G). *In vivo* $DPPG_2$-TSL showed prolonged circulation time in rodents [17,21] and cats [22]. Hence, $DPPG_2$-TSLs seem well suited for *in vivo* applications in clinical settings.

Recent release studies have noted that body fluids such as plasma or serum can have a profound effect on the transition temperature of liposomes and the efficiency of drug release [23,24]. It is thought that protein opsonization and/or penetration of proteins into the lipid bilayer are responsible for these changes, which usually shift the release temperature (T_r) to lower values by destabilizing the membrane. (Fig. 1A) [25,26]. In light of the therapeutic implications and safety issues raised by uncontrolled release, investigation of these alterations in T_r is extremely important for the translatability of *in vitro* studies into clinical practice. In addition, the formation of a protein corona can affect both the circulation time of a drug carrier and its final destination, e.g., resulting in many cases in unwanted uptake by the liver or spleen.

The standard way to determine the temperature response of a TSL formulation is to measure changes in the fluorescence intensity of released carboxyfluorescein (CF) by fluorescence spectroscopy (FS) [27,28]. This assay is based on the assumption that the concentration of CF inside the liposome is so high that the fluorescent molecules are self-quenched, and dequench only after release from the liposome [29,30].

In our assay for the quantification of release, we instead combine fluorescence correlation spectroscopy (FCS) with the use of fluorescein di-β-D-galactopyranoside (FDG). FDG is non-fluorescent, and formation of the fluorescein emitter requires the membrane-impermeable enzyme β-galactosidase (β-Gal). Thus, as long as FDG is confined within the liposomes, no signal will be detected (Fig. 2B, I). If β-Gal is added to the dispersion, free FDG is cleaved and the resulting fluorescence can be characterized, and the levels of release measured, with high sensitivity by FCS (Fig. 2B, II and III). This technique is an established method that allows one to determine not only concentrations of fluorescent particles in the pico- to nanomolar range (10^{-12}–10^{-9} M, Fig. S1), but also their diffusion coefficients, hydrodynamic radii and binding or cleavage interactions [31–36] – even in complex media like plasma or serum [37,38]. Diffusion of the fluorescently labeled particles through the confocal volume induces fluctuations in the fluorescence signal (Fig. 1B). A model fit to the time correlation of the signal yields the physical characteristics mentioned above. Its flexibility makes FCS an ideal method for characterizing TSLs, their release behavior and their interaction with proteins.

However, the potential of FCS for measurements of physicochemical parameters of TSLs and the quantification of drug release has not yet been fully exploited. Earlier publications focused on single features such as the interaction of proteins and liposomes [37,39] or the leakage or release of dyes from liposomes [40–43], but so far no study has attempted to characterize both of these processes simultaneously. However, understanding the interaction of liposomes with their protein environment and quantifying levels of unwanted release of drug molecules are vital prerequisites for the successful pharmaceutical development of targeted, liposome-based drug-delivery systems.

Here we study the effects of plasma proteins on the release temperature T_r of $DPPG_2$-TSLs and the temperature-dependent release of cargo molecules. Proteins were chosen, because $DPPG_2$-TSL were prone to stabilizing and destabilizing effects of proteins. We use FDG encapsulated in $DPPG_2$-TSLs combined with FCS to quantify the molar release ratio of FDG per $DPPG_2$-TSL for four representative plasma proteins. In a second set of experiments we use FCS to measure the binding isotherms of the four plasma proteins to $DPPG_2$-TSL and determine their equilibrium binding constants. We show that FCS is capable of quantifying the release profile in buffer and allows for measurements in blood plasma. The FCS data confirm that the temperature of release is shifted in

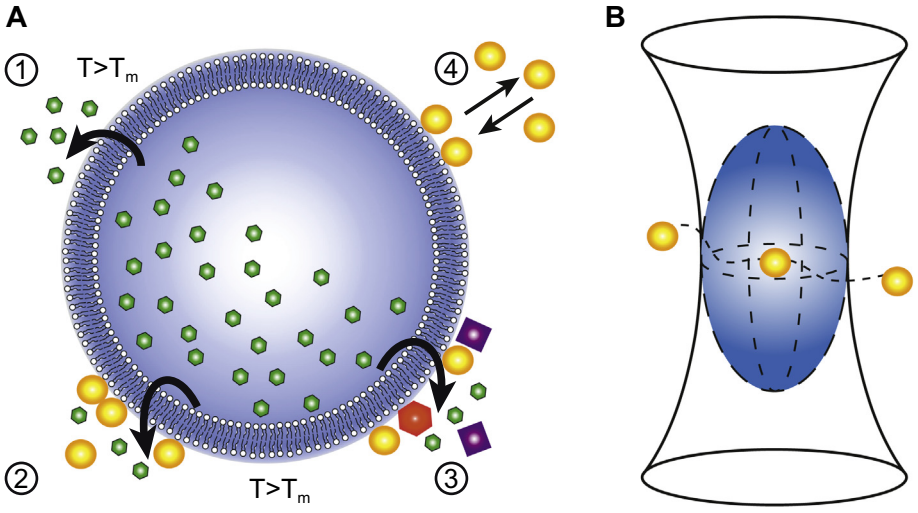

Fig. 1. (A) Pictogram that illustrates the experimental setting and the processes studied: Release of dye (green hexagons) from liposomes in buffer (1), in the presence of a specific plasma protein (2, yellow spheres) and in the presence of whole plasma (3, mixed colors). The reversible binding of selected plasma proteins to $DPPG_2$-TSL (4) was also studied. (B) The confocal volume in an FCS experiment: Fluorescently labeled particles diffuse through the confocal volume, leading to fluctuations in the fluorescence intensity (not drawn to scale), from which several physical parameters of interest, such as particle concentration and size, can be determined. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)