Investigating plasma protein adsorption for 5-fluorouracil-encapsulating liposomes

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5-Fluorouracil (5-FU) is one of the most prescribed small molecule drugs for the treatment of several tumors, including colorectal, breast, liver and pancreatic cancer [1]. However, it has a short half-life (<30 min), and its administration is usually recommended in high doses over long periods of time, which can lead to severe systemic side effects induced by its cardiac, neural, gastrointestinal and hematological toxicities. While numerous approaches have been proposed to minimize the side effects of 5-FU treatment, nanotechnological drug delivery systems have shown the most promising results in improving 5-FU's therapeutic index, although encapsulation efficiencies are low and rapid leakage is known to occur [2].

Liposomes possess several advantages as drug delivery vehicles, including prolonged circulation kinetics, passive targeting properties, as well as the possibility to encapsulate both hydrophobic and hydrophilic drugs [3]. The stability and fate of liposomes in biological environments are affected by a wide variety of factors, including lipid composition, size, surface charge, as well as the nature of their biomolecular partners, which can ultimately influence therapeutic efficiency. As with any nanostructure, upon entering a biological environment, liposomes are surrounded by a dynamic biomolecular coating of proteins, known as protein corona, which evolves over time and controls particle fate and transport properties [4] (Figure 1). As recent studies emphasize that lipid composition is one of the most influencing factors of protein corona formation on the liposomal surface [5,6], the aim of the present study is to investigate the protein adsorption behavior of 5-FU-encapsulating liposomes with various compositions. The ultimate goal of our ongoing investigation is to define an optimal 5-FU-encapsulating liposomal formulation with a rationally controlled protein adsorption pattern which ensures increased liposome circulation time and reduced *in vivo* immune recognition.

Experimental

Liposome preparation

Liposomes were generated through thin-film hydration (TFH) [7], followed by sonication and extrusion, with 5-FU loading by passive encapsulation. The lipids used in this investigation were Egg PC, DPPC and DSPC, either monocomponent or containing 40 mol% cholesterol.

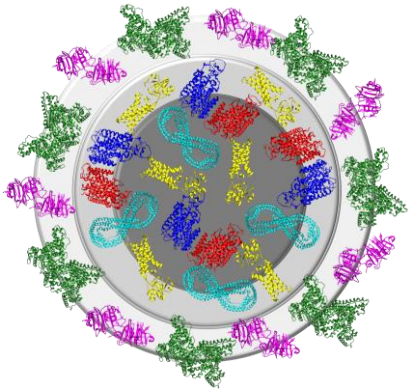

Figure 1. General structure of a liposomal protein corona. Liposome is colored in dark grey, hard protein corona with proteins with high affinity for the liposomal surface is colored medium grey, and soft protein corona with proteins with low affinity for the liposomal surface is colored light grey. Different proteins are shown as ribbon representations.

In brief, lipids were dissolved in chloroform, which was evaporated under reduced pressure. Lipid films were kept under vacuum and the system was flushed with N_2 to remove traces of organic solvent. Dry lipid films were hydrated with 10 mM PBS (pH 7.4) or 10 mM 5-FU in 10 mM PBS (pH 7.4). The final lipid concentration in the hydration solution was 7.5 mM. Liposomal suspensions were sonicated for 15 min at 26 kHz and extruded 11 times through 200 nm pore size polycarbonate membranes using a Mini-Extruder (Avanti), at a temperature greater than the phase transition temperature of the used lipid. Unentrapped drug was removed by dialysis at 4°C in 125 volumes of 10 mM PBS using a 12-14 kDa molecular weight cutoff dialysis membrane. All prepared formulations were stored at 4°C.

Vesicle characterization

Liposome size was measured through dynamic light scattering (DLS) using an Amerigo instrument (Cordouan Technologies). Zeta potential was measured using the same instrument through Laser Doppler Electrophoresis (LDE).

5-FU concentration was determined at 266 nm, on a Shimadzu UV 1900i UV-Vis spectrophotometer, using a standard curve which was linear in the 0.83-100 μM 5-FU range (R^2=0.9996). Encapsulation efficiencies (EE %) were calculated after removal of unencapsulated drug, while drug intravesicular concentrations were approximated assuming no lipid loss, with liposomes being regarded as spherical and monodisperse, with a mean bilayer thickness of 4.47 nm for monocomponent liposomes and 4.8 nm for cholesterol-containing formulations [8]. Mean cross sectional areas considered for the used lipids were 0.64 nm^2 for DPPC, 0.671 nm^2 for DSPC and 0.27 nm^2 for cholesterol [9]. Drug release behavior of liposomal formulations was investigated through dialysis at 37°C under continuous stirring.

Plasma protein adsorption

Liposomes were incubated with rat plasma for 1 h at 37°C in a 3:1 ratio (v/v) under continuous stirring. The liposome-protein corona complexes were recovered after centrifugation for 10 min at 10,000 x g at 4°C and then washed three times with cold 10 mM PBS in order to remove unbound or loosely bound proteins (soft protein corona). The adsorbed proteins (hard protein corona) were separated by one-dimensional SDS-PAGE and stained with Coomassie Brilliant Blue.

Results

Characterization of liposomal formulations

Mean vesicle diameters were in the expected range for all generated liposomal formulations, around 200 nm (Table 1). As expected, drug encapsulation efficiency was low for all tested liposomal formulations, the highest encapsulation efficiency being obtained for DPPC:Chol 60:40 mol% (Table 1). Estimated intravesicular concentrations of 5-FU were similar to ones observed by other groups [7].

Table 1. Liposomal formulation characteristics

Formulation	Mean vesicle diameter (nm)	EE (%)	Estimated intravesicular 5-FU concentration (mM)
Egg PC	174	6.58	14.82
Egg PC:Chol 60:40 mol%	186	5.97	16.46
DPPC	240	9.32	17.41
DPPC:Chol 60:40 mol%	205	10.19	29.59

Plasma protein adsorption

Liposome corona formation upon plasma incubation was first confirmed through DLS measurements. Thus, the mean hydrodynamic diameter of 5-FU-containing liposomes increased upon plasma incubation by at least a few nanometers, and varied greatly among formulations. At the same time, the zeta potential of plasma-incubated liposomal suspensions decreased in all cases, a phenomenon which is known to occur due to the overall negative charge of plasma proteins at physiological pH [6].

SDS-PAGE experiments confirmed both protein corona formation on the surface of all tested liposomal formulations, as well as different interaction strengths between proteins and the liposomal surface. As expected, protein adsorption patterns were influenced by liposomal composition, with both the main lipid constituent and cholesterol presence impacting protein preference for the liposomal surface. No difference was observed between formulations encapsulating 5-FU and the same formulations without drug, suggesting that protein adsorption patterns are not influenced by 5-FU presence. Most likely, the drug is completely distributed inside liposomes, leaving the external surface identical with that of homologous liposomes without 5-FU.

Following incubation, several protein bands which were not present in the supernatant could be observed for some liposomal formulations, suggesting the successful depletion of specific protein species from the plasma. In addition, several protein bands corresponding to abundant proteins in the plasma could be identified (e.g. serum albumin), suggesting that not only the type, but also protein plasma concentration plays a role in liposomal protein corona formation. Protein bands which appear only in the supernatant likely correspond to proteins which do not associate strongly with liposomes and do not contribute to hard protein corona formation.

Conclusions

Several liposomal formulations of 5-FU were successfully prepared through TFH, followed by sonication and extrusion. Although encapsulation efficiency was low, estimated intravesicular 5-FU concentrations were in the millimolar range for all generated liposomal formulations.

The plasma protein adsorption patterns identified in our study demonstrate that lipid composition has a major impact on the preference of plasma constituents for the nanovehicle surface. Our work in this direction will be continued with the identification of the differentially adsorbed proteins through LC-MS, as well as measuring 5-FU concentration in plasma following incubation.

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