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Correlation of structure and echogenicity of nanoscaled ultrasound contrast agents *in vitro*

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ABSTRACT

Ultrasound is a common tool for clinical diagnosis due to its safety and economic. Especially the addition of ultrasound contrast agents leads to a high diagnostic reliability. In recent years ultrasound has been used as a trigger for directed drug delivery or to enhance thrombolysis. We developed a nanoscaled ultrasound contrast agent (NUSCA) to improve these applications. In the future drugs can be incorporated into this contrast agent to achieve a combination of ultrasound diagnosis and therapy. The aim of the present study is to elucidate the structure of the nanoscaled lipid formulations and a potential dependence of the ultrasound contrast enhancement on this structure. Our NUSCA is based on the phospholipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and the single-chained polyethylene glycol (40) stearate (PEG40S). In this study the effect of increasing concentrations of the single chained PEG40S on the structure of the lipid formulations was characterised using Dynamic Light Scattering, cryo-Transmission Electron Microscopy, Nuclear Magnetic Resonance spectroscopy, lipid monolayer studies and epifluorescence measurements. In addition, the ultrasound contrast enhancement for the formulations was determined *in vitro*. Dependence between structure and ultrasound contrast was found. All PEG40S concentrations lead to a mixture of liposomes and discoid micelles. With increasing PEG40S content the amount of micelles increased. Certain PEG40S concentrations lead to an ultrasound contrast superior to the contrast of the commercially available ultrasound contrast agent SonoVue®.

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1. Introduction

The use of ultrasound for sonothrombolysis or targeted drug and gene delivery is a growing field in medical research [1–3]. Ultrasound is able to increase the cellular uptake of drugs and genes especially after the addition of ultrasound contrast agents based on gas bubbles. Juffermanns et al. demonstrated cell membrane deformation and transient pore formation through oscillating microbubbles near the cell surfaces [4,5]. Commercially available ultrasound contrast agents like SonoVue® or Optison™ are based on gas filled and shell stabilised microbubbles [6,7]. These microbubbles are too big to penetrate into tumour cells or blood clots to achieve a directed drug delivery. Therefore we developed a new nanoscaled ultrasound contrast agent (NUSCA) [8] into which thrombolytic drugs shall be incorporated in future studies. Thus we want to achieve a combination of ultrasonic diagnosis and thrombolytic therapy. Our nanoscaled contrast agent is based on lipid formulations of the double-chained phospholipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and the single-chained polyethylene glycol (40) stearate (PEG40S).

We showed for the first time a brilliant ultrasound contrast for NUSCA in a size range of 100–300 nm [8]. The contrast intensity remained constant for 5 min whereas the contrast intensity of

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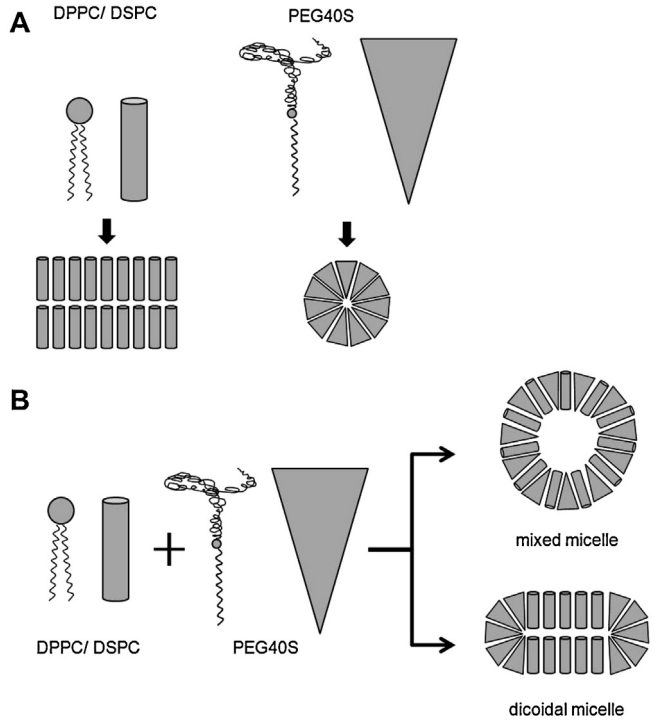

Fig. 1. Molecular shape and idealised organisation of DPPC, DSPC and PEG40S. (A) Pure substances, (B) equimolar mixtures of DPPC/DSPC and PEG40S.

SonoVue® decreased after 2 min in our *in vitro* model. We proved further that this contrast is stable for at least 4 weeks. In the present study our aim was to optimise the ultrasound reflectivity and to find an explanation for the brilliant ultrasound contrast. A possible explanation for the good ultrasound contrast is the formation of liposomes and micelles due to the mixing behaviour of our compounds. Therefore lipid formulations with different molar ratios of DPPC and PEG40S or DSPC and PEG40S were prepared to elucidate the structure of this NUSCA. This structure is very important for the further use of our NUSCA. A formation of micelles, liposomes or a mixture of both seems to be likely. Micelles are suitable for the encapsulation of lipophilic drugs. However, hydrophilic thrombolytic drugs *e.g.* urokinase or recombinant tissue plasminogen activator (rt-PA) can only be loaded into liposomes. For a combination of ultrasonic diagnosis of the occluded blood vessel and the thrombolytic therapy an excellent echogenicity is as essential as a high drug loading capacity. Therefore the PEG40S concentration has to be properly adjusted.

Several studies have been undertaken to elucidate the structure and phase behaviour of double-chained polyethylene glycol lipids (PEG-lipids) in mixtures with different phospholipids [9–11]. Mixtures of DPPC or DSPC with the correlating PEG-lipids form micelles beside liposomes at low concentrations of PEG-lipids. Higher concentrations lead to the formation of micelles only with decreasing size at increasing PEG-lipid concentration [9]. The behaviour of our single-chained PEG40S cannot be equated with the behaviour of the double-chained PEG-lipids. In Fig. 1A the molecular shapes and the idealised organisation of DPPC, DSPC and PEG40S can be seen. Compared to PEG-lipids PEG40S shows a smaller hydrophobic part which favours the formation of small micelles [12,13]. In mixture with the liposome forming phospholipids DPPC or DSPC a formation of liposomes at low molar ratios of PEG40S may be possible. Higher molar ratios should lead to the formation of homogenous mixed micelles or phase separated discoid micelles (Fig. 1B). Also the formation of pure PEG40S micelles beside DPPC or DSPC liposomes is possible if the compounds are immiscible. We utilised lipid monolayer studies using a Langmuir film balance to check the miscibility of surface active molecules. Comparing the lateral pressure *vs.* area per molecule isotherms (Π–A isotherms) of the pure phospholipids, the pure PEG40S and their mixtures enables us to estimate the miscibility of our compounds.

In this study we combine two methods to determine the contrast agent's size: Dynamic Light Scattering (DLS) and cryo-Transmission Electron Microscopy (cryo-TEM). Further structural information was gathered with cryo-TEM and Phosphorus Nuclear Magnetic Resonance spectroscopy (^{31}P-NMR). Lipid monolayer studies and epifluorescence measurements were carried out on a Langmuir film balance combined with a fluorescence microscope to gather information concerning miscibility of the compounds as described above. To correlate the ultrasound contrast and the structure of the NUSCA the echogenicity of all the formulations was measured in an *in vitro* closed-loop flow system.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Lipoid GmbH (Ludwigshafen, Germany), polyethylene glycol (40) stearate (PEG40S) from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Stock solutions of the lipids were prepared in a mixture of chloroform:methanol (2:1, v:v) and stored at 4 °C. Chloroform (HPLC-grade) and methanol (HPLC-grade) were purchased from Fisher Scientific (Loughborough, UK). All other chemicals were of analytical grade and were used as received.

2.2. Preparation of lipid formulations

The lipid formulations composed of DPPC and PEG40S or DSPC and PEG40S with different molar ratios (see Table 1) were prepared using the thin film hydration method [14,15]. Briefly, from a stock solution (chloroform:methanol, 2:1) the lipids were mixed in a round bottom flask to a total amount of 10 mg lipid. The lipid mixture was subsequently dried to a lipid film using a rotary evaporator (Heidolph Laborota 4000 efficient, Heidolph Instruments, Schwabach, Germany) under vacuum at 40 °C. The resulting film was rehydrated with 1 ml of phosphate-buffered saline (PBS) pH 7.4 (0.15 mol/l). After vigorous shaking the lipid dispersions were sonicated in a bath-type sonicator (Bandelin Sonorex RK 100H, Bandelin Electronics, Berlin, Germany, maximal energy) for 20 s at 55 °C (DPPC/PEG40S) or 65 °C (DSPC/PEG40S). After incubation for 60 min at the above mentioned temperatures the dispersions were again sonicated in the bath-type sonicator for 2 min. Immediately after this the warm lipid formulations were sonicated with a probe sonicator (Branson Sonifier 250, G. Heinemann Ultraschall-/Labortechnik, Schwäbisch Gmünd, Germany) for 20 s, using maximum ultrasound output level and a duty cycle of 20% at the air–water interface. After cooling to room temperature the lipid dispersions were transferred into microtubes leaving the produced foam in the flask and stored at 4 °C.

2.3. Dynamic Light Scattering

The hydrodynamic diameter of lipid formulations, prepared and stored as described above, was determined by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) equipped with a 10 mW HeNe laser at a wavelength of 633 nm and a temperature of 25 °C, as described previously [16]. Scattered light was detected at a 173° angle with laser attenuation and measurement position adjusted automatically by the Malvern software. The particle size is calculated automatically

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