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Preparation of small amounts of sterile siRNA-liposomes with high entrapping efficiency by dual asymmetric centrifugation (DAC)

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ABSTRACT

Liposomal formulation of siRNA is an attractive approach for improving its delivery *in vivo*, shielding the RNA from nucleases and promoting tumor targeting. Here, the production of very small batch sizes of siRNA-liposomes by using the "dual asymmetric centrifugation (DAC)" technique was investigated. This new technique combines rapid and sterile liposome preparation with very high entrapping efficiencies. DAC is here presented in conjunction with a non-destructive microscale analysis based on double fluorescence labeling, which enables monitoring of siRNA integrity during the liposomal preparation. Integrity is reflected in spatial proximity of the dyes, which results in measurable fluorescence resonance energy transfer (FRET). The combination of DAC and the sensitive FRET analysis allows the handling of batch sizes down to 20 mg of conventional liposomes (CL) and sterically stabilized liposomes (SL). These were prepared in common 2 ml reaction tubes and loaded with calcein or labeled siRNA. Liposome sizes were 79±16 nm for CL and 109±9 nm for SL loaded with siRNA. Trapping efficiencies ranged from 43 to 81%, depending on batch size, enclosed compound, and liposome composition. FRET monitoring showed that the siRNA remained intact throughout DAC and that liposomal formulations protected the siRNA from nucleases. siRNA-liposomes remained stable for at least 3 months.

1. Introduction

Soon after its discovery by Fire and Mello in 1998 [1], RNAi has evolved into a major subject in all areas of life sciences. Acting as a source of antisense RNA that can induce the degradation of multiple copies of a targeted mRNA small interfering RNAs (siRNAs) can decrease protein expression very efficiently. The potential use of the natural, programmable amplification mechanism of RNAi has led to a renewed surge in drug research in the antisense field, and the chemical and pharmaceutical knowledge obtained in decades of antisense research has quickly taken siRNA to clinical trials [2]. Typical siRNAs are 19–21 nt long siRNA duplexes with a two-nucleotide overhang on the 3′-end of each strand [3].

Because nucleic acids like siRNA carry multiple negative charges, cell membrane passage to achieve cell penetration has to be aided [4]. The addition of cationic lipids, which form structurally ill-defined lipoplexes with the siRNAs (lipoplexes) [5], has been especially successful in cell culture. Application of lipoplexes in vivo is more challenging, mainly because the transfection agents show inflammatory properties incompatible with the demands of drug development [4,6]. Thus, some publications on possible formulations of siRNA make mention of better defined lipid nanoparticles like liposomes as possible vehicles that offer protection against bloodstream nucleases as well as unique targeting properties: a definite milestone in the field, Zimmermann et al. reported the successful application of stable nucleic acid lipid particles (SNALPS)–liposome like structures containing 40% cationic lipids–for knockdown of endogenous apoB mRNA in primates [7]. Other reports describe formulations containing neutral lipids and siRNA (targeting Interleukin 8 [8], focal adhesion kinase [9] and tyrosine kinase receptor EphA2 [10]) were allegedly liposomes, but there is ambiguity about the exact nature and homogeneity of the resulting particles.

Liposomes in the proper sense are spherical vesicles in which an inner aqueous volume is surrounded by one or more phospholipid bilayers [11]. A liposomal preparation suitable for in vivo application is typically characterized by (i) its average size in the double to lower triple-digit nanometer range [12–16], (ii) a size distribution which is not too broad (polydispersity index (PI) below 0.6) [12] and (iii) in most cases neutral surfaces [12]. The entrapment of a drug in liposomes offers two major advantages: protection of the drug and a resulting prolongation of its circulation time [17,18], as well as

Abbreviations: siRNA, Small interfering RNA; RNAi, RNA interference; nt, nucleotides; SL, sterically stabilized liposomes; CL, conventional liposomes; DAC, dual asymmetric centrifugation; PI, polydispersity index; VPG, vesicular phospholipid gel; EE, entrapment efficiency; CRP, C-reactive protein; AUC, area under the curve; FL, fluorescein; TMR, tetramethylrhodamin; FRET, Fluorescence resonance energy transfer; EPR, enhanced permeability and retention; PP, polypropylene; PEG, polyethylene glycol.

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accumulation in tumor tissue due to the enhanced permeability and retention effect (EPR-effect) [12,19–21].

For *in vivo* applications, two types of liposomes have so far been proven suitable for clinical use. The first type are plain liposomes consisting of fully hydrogenated phosphatidylcholine [22,23] (from egg or soy) and cholesterol. While the phospholipids are the basic components of the liposome bilayers, cholesterol in molar ratios of minimal 33% reduce lipid exchange with lipoproteins and control the fluidity of the membranes [24]. Such liposomes, also called conventional liposomes (CL), feature a neutral surface, which avoids binding of various serum proteins like antibodies, complement factors or CRP to a certain extent (e.g. less opsonisation) and thus, reduce uptake by macrophages [12].

The second type of clinically relevant liposomes are sterically stabilized liposomes (SL) which are also called Stealth liposomes [25–28]. SL are derived from CL by the addition of hydrophilic polymers to the surface of the liposome membranes, a feature which further reduces binding of serum proteins (e.g. very little opsonisation). Hence, the uptake of the liposomes by macrophages is further diminished. As hydrophilic polymers, polyethylene glycol (PEG)-chains chemically bound to phospholipids (as a membrane anchor) are used.

siRNA as a water soluble compound is suitable for entrapment in the inner aqueous core of CL's and SL's in high amounts. Such liposomal entrapment of siRNA is expected to be stable, because the charge (poly-anion) and the size of a RNA molecule impede passive diffusion across the liposome bilayer.

Classical liposome fabrication techniques, such as extrusion [29], high pressure homogenization [30] ultrasound treatment [31], or detergent dialysis [32] present various drawbacks in the formulation of siRNA containing liposomes: Because synthetic siRNAs are very expensive, there is a strong incentive for reducing the batch size of liposomal siRNA preparations, *e.g.* in screening for optimal lipid composition. At the same time the typical demands in drug development must be considered, including sterility, analytics, and validation issues. The well known techniques mentioned above fail to offer a suitable combination of features such as sterility, small batch sizes, and high entrapment efficiencies. Since this combination is, however, highly desirable for siRNA entrapment [12,30,33] we decided to explore the use of a new, recently described technique [34], which principally allows to prepare very small batches of sterile liposomes with high entrapment efficiencies for water soluble compounds—the "dual asymmetric centrifugation" (DAC) (Fig. 1). Liposome formation by DAC is facilitated by a kind of homogenization of highly concentrated lipid dispersions in a vial. DAC-homogenization takes place when the concentrated, viscous lipid dispersion is centrifuged around a central axis (axis 1) while the vial itself turns around its own vertical axis (axis 2). While the viscous lipid dispersion is accelerated away from axis 1 due to centripetal forces (outwards direction), the material is moved inwards due to friction of the material to the wall of the constantly turning vial. Both overlaying movements of the viscous lipid dispersions result in its homogenization and in the formation of a highly viscous liposome formulation (vesicular phospholipid gel, VPG).

In this study we systematically investigated the preparation of very small batch sizes of sterile siRNA containing liposomes (SL and CL) with high trapping efficiencies by DAC. For this, various vials, homogenization aids and lipid mixtures were investigated and the structural integrity of the entrapped siRNA was monitored by a non-destructive method based on fluorescence resonance energy transfer (FRET).

2. Materials and methods

2.1. siRNA

Dye-siRNA constructs labeled with fluorescein (FL) and tetramethylrhodamine (TMR) were purchased from IBA GmbH (Göttingen, Germany). Their spectral characterization is reported in [35]. The siRNA sequence is directed against the EGFP protein (GenBank Accession #U55762) (sense: 5′-GCAAGCUGACCCUGAAGUUCAdC-FL-3′ and antisense 5′-TMR-GCCGUUCGACUGGGACUUCAAG-3′).

2.2. Preparation of ds siRNA sample

For the formation of siRNA duplexes, sense and antisense strand solutions were mixed in a ratio of 1:1.1 as stated in earlier studies [35] and PBS-stock solution ('10×PBS') was added (Gibco, Invitrogen Corp, Carlsbad, CA, USA) ('10xPBS'-volume: 1/10 of the volume of the siRNA-mixture) to get a physiologic PBS-solution (1×PBS) containing 43 μM of the sense and 47 μM of the antisense strands. The siRNA was annealed for 1 h at 37 °C after 5 min separation at 90 °C. siRNA duplex solutions were stored at −20 °C.

2.3. Preparation of dry phospholipid mixtures

For preparing conventional liposomes (CL) a molecular dispersed mixture of hydrogenated egg phosphatidylcholin (EPC3) and cholesterol (Chol) (55:45 molar ratio) were used (Lipoid GmbH; Ludwigshafen, Germany). A molecular dispersed mixture of EPC3 and Chol in a molecular ratio of 67:33 was prepared by dissolving the lipids in EtOH and removal of the solvent in a vacuum centrifuge (SpeedVac; Savant Sc110; GMI Inc, Ramsey, MI, USA).

For preparing sterically stabilized liposomes (SL) a molecular dispersed mixture of EPC3, Chol and MPEG-2000-DSPE (Lipoid GmbH) in a molar ratio of 56.9:37.9:5.2 were used. The lipids were dissolved

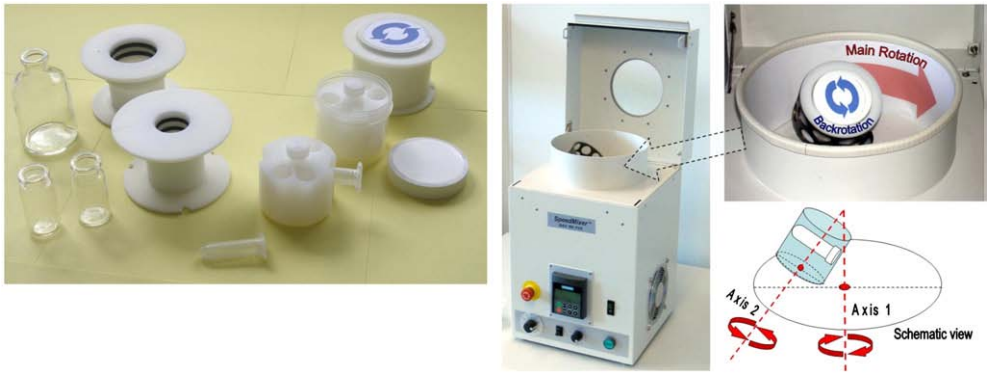

Fig. 1. DAC-principle and -equipment. On the right: Dual asymmetric centrifuge (DAC) with rotation orientations indicated and a schematic drawing of the DAC principle. The drawing shows a vial in the "unusual" horizontal orientation (see Results/Discussion). On the left: suitable glass vials and reaction tubes for DAC as well as the respective vial holders/spindles (left: glass injection vials and vial holders, right: reaction tubes and vial holders for horizontal positioning of the reaction tubes (plus container and spindle)).