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Lipid nanoparticle purification by Spin Centrifugation–Dialysis (SCD): A facile and high-throughput approach for small scale preparation of siRNA–lipid complexes

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ABSTRACT

This paper describes the use of Spin Centrifugation–Dialysis (SCD) for small-scale concentration/purification of siRNA–lipid complexes designed for use as therapeutic agents for gene silencing. SCD consists of a two-step method for concentration, filtration and buffer exchange of Lipid Nanoparticles (LNP) to provide a homogeneous preparation suitable for injection. Here, we compare SCD with the more traditionally used Tangential Flow Filtration (TFF), and demonstrate the physicochemical and biological comparability of LNPs produced with both methods. TFF is a highly scalable method used in both developmental and production applications, but is limited in terms of miniaturization. In contrast to TFF, SCD is faster, less expensive, and requires less oversight for assembling LNPs for small-scale applications, such as target screening both *in vitro* and *in vivo*. The finding that SCD is a viable method for filtering LNPs in a manner similar to TFF, producing particles with comparable properties and biological activity, is significant given the complexity and sensitivity of LNPs to processing conditions.

1. Introduction

RNA interference is a naturally occurring gene silencing mechanism in eukaryotes that controls the expression of endogenous genes (Dorsett and Tuschl, 2004). Synthetic siRNAs can be designed to take advantage of the RNA-induced silencing complex pathway to silence genes of interest. In order to leverage this approach for clinical therapeutics, a delivery vehicle is required to facilitate cellular uptake. Cationic liposomes have been widely used to deliver siRNAs effectively due to their ability to associate with cell membranes and promote release of siRNA from endosomes (Li and Szoka, 2007). Delivery efficiency of the liposomes is dependent upon the lipid composition, lipid to nucleic acid (N/P) ratio, particle size, and formulation method used to assemble and purify them. Lipid Nanoparticle (LNP) is a type of liposomal drug carrier that serves as an effective delivery vehicle for siRNA to the liver (Zimmermann et al., 2006).

A scalable, extrusion-free method for preparation of liposomes has been published previously (Jeffs et al., 2005), where the LNPs are formed by mixing the siRNA (in aqueous buffer) and lipids (in ethanol) at a controlled speed, followed by dilution with buffer in a step-wise manner. After the initial particle assembly steps, the formulation typically must be further processed to concentrate, remove solvent, and perform buffer exchange. Many downstream processing methods are described in the literature, and Tangential Flow Filtration (TFF) (Pattnaik and Ray, 2009), a highly scalable method, is often utilized. TFF, however, is not ideally suited for high throughput small scale preparation of lipid nanoparticles. Other techniques for filtration/purification of liposomes by centrifugation have been reported previously (Xu et al., 1999; Mortazavi et al., 2007). For example, dual asymmetric centrifugation (DAC) is used for assembling LNPs, but has only shown 81% encapsulation for siRNA–liposome complexes (Hirsch et al., 2009). Here we describe an easy, robust, high-throughput, and inexpensive technique for preparing highly homogeneous LNPs using equipment commonly found in research labs (flowchart shown in Fig. 1). We have used, in place of TFF, a highly versatile spin centrifugation and dialysis (SCD) step. Along with the impinging jet mixing method, this approach provides a facile method for supporting small-scale preparation of LNPs for *in vitro* transfection and rodent studies, and is capable of producing LNPs with even higher entrapment efficiency (98%) over different lipid compositions.

We have used two types of cationic liposomes to test the versatility of the SCD approach – *LNP05* (Tao et al., 2011) and

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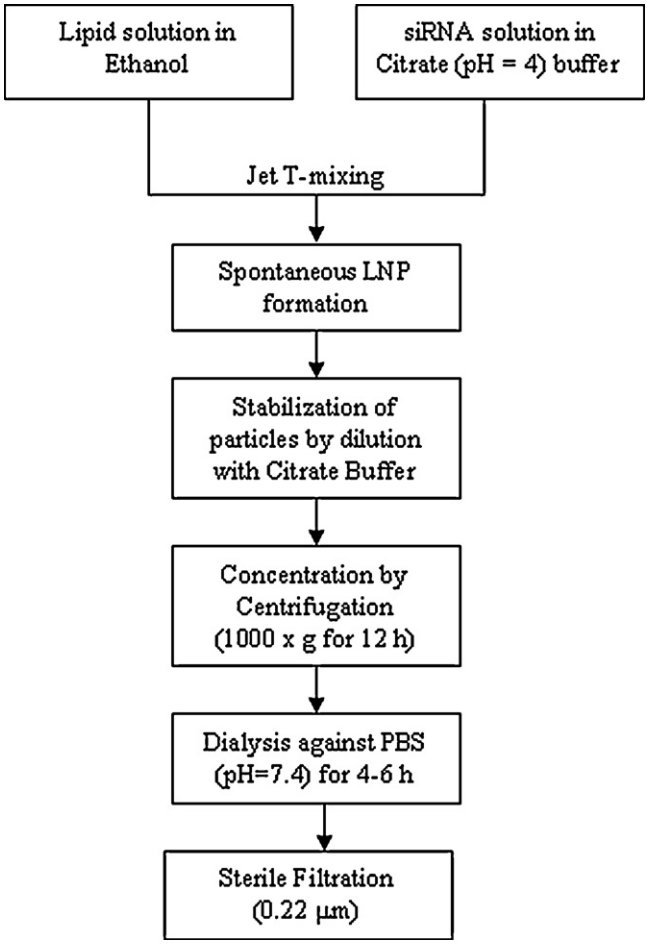

Fig. 1. Flowchart for the preparation of LNPs.

DlinDMA-SNALP (Zimmermann et al., 2006). These two compositions differ in the N/Ps (3.3 and 6.0 respectively) and morphology. The LNP05 formulations are composed of Octyl CLinDMA, cholesterol, and PEG-DMG. The DlinDMA-SNALP system contains DlinDMA, cholesterol, DSPC and PEG-C-DMA. The structures of lipids are shown in Fig. 2.

To test both SCD and TFF approaches head to head, three carbonyl reductase 4 (Cbr4) siRNAs (targeting different sites of the mRNA) were formulated in LNP05. These siRNAs were previously shown to have a differential potency *in vitro* and *in vivo* (internal data). The upstream mixing of the siRNA and lipid stock solutions remained common for both approaches: the diluted product was split into two batches, one processed using TFF, and the other processed *via* SCD for final concentration and purification. Thus, allowing head to head comparison of the two downstream processes. The final concentrated and filtered LNPs encapsulating these siRNAs were then fully characterized and tested *in vitro* and *in vivo*. In order to validate the potency and duration equivalence of the LNPs, we also performed an *in vivo* imaging study in luciferase mice where we looked for sustained inhibition of luciferase expression over a period of 15 days using the LNP05 and DlinDMA-SNALP compositions.

2. Methods

2.1. Preparation and analysis of LNPs (LNP05 and DlinDMA-SNALP)

The siRNA sequences were designed (refer to Supplementary data) using a previously described algorithm (Jackson et al., 2003), and were annealed in equimolar amounts of complementary passenger and guide strands. These duplexes were encapsulated using LNP05 and DlinDMA-SNALP compositions by jet mixing the siRNA and lipid solutions, using a protocol similar to what has been published (Jeffs et al., 2005). The diafiltration step was performed using both the TFF and the SCD approaches for comparison. The spin centrifugation step was carried out using Vivaspin centrifugal concentrator tubes, spun @ 1000 × *g* for 12 h. The concentrate was then resuspended and dialyzed against PBS (pH = 7.4) for a minimum of 4 h. The samples were finally passed through a 0.22 μm sterile filter and stored at 4 °C under argon (for more details refer to the Supporting Data). The siRNA and lipids were quantified by HPLC. Encapsulation was determined using a SYBR™ Gold fluorescence assay measured at λ_{ex} = 495 nm and λ_{em} = 535 nm. The particle size and polydispersity were measured with a Brookhaven dynamic light scattering instrument.

2.2. In vitro and in vivo gene silencing and duration

In vitro transfections of LNPs were performed in Hepa1,6 cells and gene silencing was quantified as described previously (Strapps et al., 2010). For *in vivo* studies, C57BL/6 mice were dosed with 3 mpk Cbr4 siRNA sequences (in LNP05) and sacrificed at 3 days.

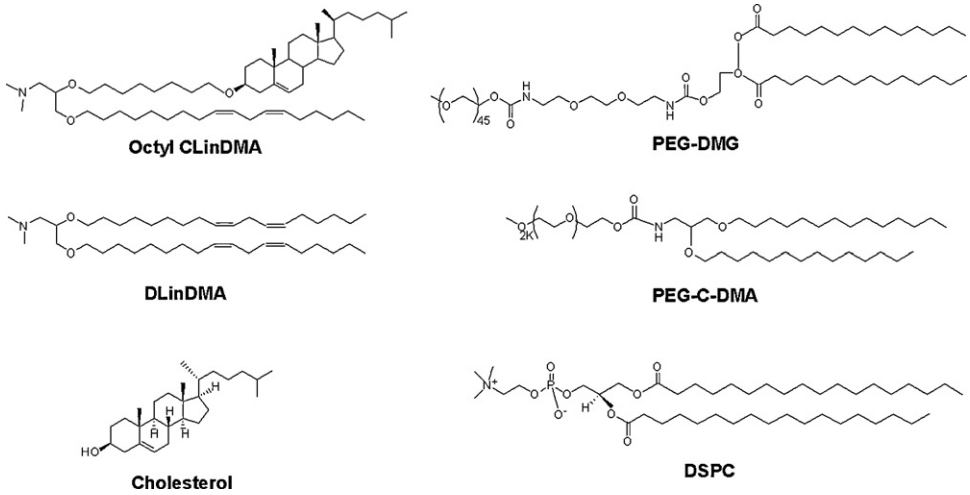

Fig. 2. Chemical structures of lipids.

Table 1
Analytical and *in vitro* potency data for LNPs.

Sample ID	Lipid composition	siRNA (mg/mL)	N/P	Encapsulation (%)	Particle size (nm)	Polydispersity	IC50 (nM)
Cbr4_596_TFF	LNP05	1.8	3.2	90	89	0.16	2.6
Cbr4_596_SCD	LNP05	1.5	3.3	96	101	0.10	1.5
Cbr4_328_TFF	LNP05	1.8	3.4	91	85	0.16	3.9
Cbr4_328_SCD	LNP05	1.9	3.0	98	95	0.11	3.1
Cbr4_731_TFF	LNP05	1.7	3.2	93	91	0.15	1.9
Cbr4_731_SCD	LNP05	1.9	3.2	97	104	0.11	1.7
Luc_80_TFF	DlinDMA-SNALP	1.1	7.1	95	67	0.18	ND[a]
Luc_80_SCD	DlinDMA-SNALP	1.7	6.9	96	74	0.07	ND[a]

[a] Not determined.

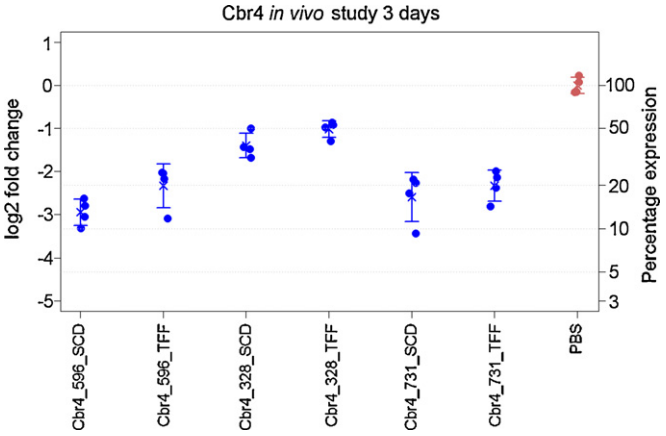

Fig. 3. *In vivo* mRNA knock down of Cbr4 demonstrating equivalence of SCD and TFF approaches.

Cohorts for controls and each siRNA tested consisted of groups of five mice. RNA isolation and quantification were done according to protocols previously established (Strapps et al., 2010). mRNA knockdown was calculated relative to a non-targeting control siRNA in each experiment. The *in vivo* duration studies were performed in homozygous Rosa26-LSL-luciferase transgenic mice (Safran et al., 2003), injected intravenously with a single dose of 3 mpk of the LNP05 and DlinDMA-SNALP containing the anti luciferase sequence (luc_80), imaged for luciferase expression on day 0 and then every 2 days until day 15.

3. Results

As illustrated in Table 1, the characterization data for LNPs made *via* SCD and TFF fall within expected ranges, indicating analytical comparability of the particles. The siRNAs were efficiently encapsulated, with values higher than described by other methods. The N/P ratios were 3.3 ± 0.3 for all the LNP05 compositions and 6.0 ± 1.2 for the DlinDMA-SNALP compositions, within an acceptable range of target values. The particle sizes remained at 100 ± 5 nm and 70 ± 4 nm for LNP05 and DlinDMA-SNALP respectively with low polydispersity index indicating a highly homogeneous composition.

Based on the IC50s observed in the *in vitro* assay, particles made *via* both approaches showed similar potency (Table 1). These LNPs were further evaluated *in vivo* by assessing the mRNA knockdown activity at day 3 in C57BL/6 mice. For each sequence, mRNA knockdown was comparable between the batches prepared by TFF and SCD (Fig. 3). In addition, a duration study in luciferase mice with LNP05 and DlinDMA-SNALP preparations tested head to head (Fig. 4) demonstrated that the percentage reduction in luciferase expression was comparable between preparation methods, with both showing sustained knockdown over 15 days following a single 3 mpk dose.

We performed statistical analysis, Wilcoxon/Mann–Whitney test, using the statistical toolbox in Matlab, on the *in vitro* data (IC50 and maxKD) as well as on our analytical parameters on a subset of 10 LNPs. At the default 5% significance level, the test failed to reject the null hypothesis of the zero median in the difference indicating that the two methods, TFF and SCD, produced particles that were not statistically different in either their biophysical parameters or *in vitro* potency.

4. Discussion

The results of analytical and biological evaluations clearly demonstrate that SCD can be used to assemble highly homogeneous LNPs suitable for *in vitro* and *in vivo* applications, and that it is a viable small-scale alternative to TFF. The critical parameters for SCD had to be carefully optimized: the optimal spin rate and the time of centrifugation required to concentrate/filter the LNPs

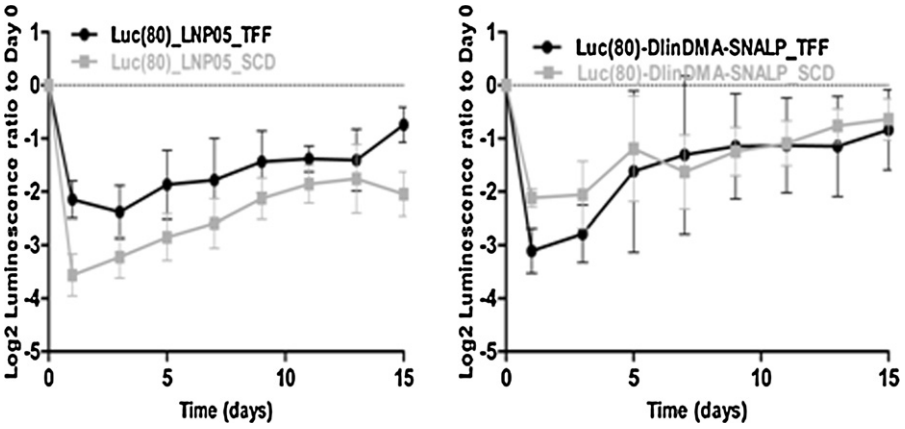

Fig. 4. Duration of luciferase expression over 15 days for LNP05 and DLinDMA-SNALP.

completely, and the dialysis time required to remove the residual ethanol. The particle sizes were monitored throughout the process to ensure there was no aggregation.

Compared to SCD, TFF is more complex to set up, and the concentration/buffer exchange step requires more time and active monitoring. Another advantage of SCD over the TFF approach is the cost savings in terms of consumables needed; SCE enables a reduction of 5–10 fold compared to TFF, which can make the screening of siRNAs more affordable and optimal. With recent interest in novel siRNA modifications to improve target specificity, siRNAs have become more expensive to synthesize, and therefore a means of miniaturizing preparation to reduce material needs is necessary. SCD is capable of formulating LNPs with only 4 mg of the starting siRNA with 85–90% yield, whereas losses from TFF are generally higher at small scales. In this study we have demonstrated the use of this approach for small scale preparations, but the process can be easily scaled up for bigger batches by using larger centrifuge tubes. Last but not the least, this approach also has the potential to be automated in order to obtain high throughput preparations of LNPs for purposes of screening siRNAs and lipids, both *in vitro* and *in vivo*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.08.017.

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