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Rapid communication

Lyophilised ready-to-use formulations of PEG-PCL-PEI nano-carriers for siRNA delivery

Thomas Endres, Mengyao Zheng, Moritz Beck-Broichsitter, Thomas Kissel*

Department of Pharmaceutics and Biopharmacy, Philipps-Universitaet Marburg, Ketzerbach 63, 35037 Marburg, Germany

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ABSTRACT

The purpose of the present study was to transfer aqueous PEG-PCL-PEI nano-suspensions into dry ready-to-use formulations, suitable for delivery of siRNA. Therefore, freshly prepared nano-suspensions were lyophilised with glucose as lyoprotectant. Firstly, the required glucose concentration for sufficient stabilisation of unloaded carriers was determined via dynamic light scattering. Morphology of fresh and rehydrated carriers was visualised by cryogenic scanning electron microscopy. Subsequently, the feasibility of siRNA loading before and after lyophilisation was investigated. For both strategies complex diameter and *in vitro* transfection efficiency were determined and correlated to freshly prepared samples. Hydrodynamic diameter (95.2 ± 1.4 nm) and size distribution (0.132 ± 0.019) of unloaded nano-suspension were restored after rehydration by addition of $\geq$1.5% of glucose before lyophilisation. Moreover, after loading of rehydrated carriers with siRNA, no significant difference in complex size was observed as compared to freshly prepared ones. Stabilisation of pre-formed carrier/siRNA complexes during lyophilisation is feasible at elevated N/P (e.g. 20) and glucose concentrations above 5%. As determined via real-time-PCR, lyophilised samples were as active as freshly prepared ones regarding transfection efficiency. In conclusion, lyophilisation is an effective technique to produce physically stable PEG-PCL-PEI formulations. These general findings may be applicable to further particulate gene delivery systems to shelf ready-to-use formulations.

In recent years many nonviral gene delivery vehicles, such as liposomes, micelles, polyplexes, solid particles or intermediate structures were widely investigated (Jeong et al., 2011). Most of these formulations basically consist of aqueous colloidal systems, suffering from inherent thermodynamic instability, subsequently leading to aggregation over time (Brus et al., 2004). This unstable nature of colloidal systems necessitates preparation immediately before administration and hampers reproducibility as well as their clinical application. Lyophilisation, offering the opportunity of dry storage and *in situ* sample rehydration, has been employed for particles (Chan et al., 2009), liposomes (Kundu et al., 2011) or polyplexes (Brus et al., 2004) with encouraging results. In a vast majority of colloidal systems, carrier diameters could be completely restored, if proper lyoprotectants in adequate lyoprotectant/nano-carrier ratios were used. Applicable additives were typically sugars (e.g. glucose, sucrose and trehalose) (Abdelwahed et al., 2006), whereas the sugar type seems to be of lesser importance (Wieber et al., 2011). Protectants in general limit mechanical damage upon freezing and carriers are incorporated in a glassy matrix, which inhibits nano-carrier aggregation (Packhaeuser et al., 2009). However, full recovery of physicochemical properties does not ensure transfection efficiency (Hahn et al., 2010). It is well known, that structural changes during lyophilisation of DNA (Poxon and Hughes, 2000) and PEI/DNA complexes (Hahn et al., 2010) may possibly decrease transfection efficiency and siRNA is even more susceptible to degradation than DNA (Gary et al., 2007). By contrast, there are examples of successfully regaining transfection efficiency after freeze-drying (Brus et al., 2004; Yadava et al., 2008).

A promising class of multifunctional gene delivery carriers is based on cationic amphiphiles (Mao et al., 2011). In aqueous media block copolymers, consisting of poly(ethylene glycol) (PEG), poly(ε-caprolactone) (PCL) and poly(ethylene imine) (PEI), self-assemble spontaneously to core–shell structured nano-carriers. Core-forming biodegradable PCL segments, which could serve as reservoirs for water-insoluble drugs or dyes, are surrounded by hydrophilic PEG moistures, maintaining suspension stability. Cationic PEI segments are amenable for complexation with nucleic acids (e.g. siRNA) (Endres et al., 2011).

It was the aim of the present study to transfer aqueous PEG-PCL-PEI nano-suspensions into dry ready-to-use formulations, suitable

* Corresponding author. Tel.: +49 6421 28 25881; fax: +49 6421 28 27016.
E-mail addresses: kissel@mailer.uni-marburg.de, kissel@staff.uni-marburg.de (T. Kissel).

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for delivery of siRNA. Therefore, freshly prepared suspensions were lyophilised together with glucose (glc) as lyoprotectant. Firstly, freeze-drying of unloaded carriers was investigated as a function of glc concentration (c_{glc}) before and after lyophilisation and rehydration by means of dynamic light scattering (DLS). Morphology was visualised by cryogenic scanning electron microscopy (cryoSEM). In addition to lyophilisation of unloaded carriers, the feasibility of siRNA loading before and after lyophilisation and rehydration was investigated at different polymer/siRNA ratios (the ratio is generally referred as PEI nitrogen per nucleic acid phosphate: N/P). Diameters and *in vitro* transfection efficiency of complexes assembled with siRNA before or after lyophilisation and rehydration were determined and compared to freshly prepared samples.

PEG-PCL-lPEI triblock copolymer was synthesised and characterised as described in detail elsewhere (Endres et al., 2011). Block length for PEG, PCL and PEI are 500, 10,000 and 2500 Da, respectively. 2′-O-methylated 25/27mer DsiRNA targeting Firefly Luciferase was purchased from Integrated DNA Technologies (IDT, Leuven, Belgium). QuantiFast™ SYBR™ Green PCR Kit, Hs_GAPDH_primer and Hs_β-actin-primer were provided by Qiagen (Hilden, Germany). Lipofectamine™ 2000 (LF) was obtained from Invitrogen (Karlsruhe, Germany).

Nano-carriers were prepared by solvent displacement (Beck-Broichsitter et al., 2010). For complexation, the appropriate amount of siRNA was added to an aliquot of nano-suspension (NS) in one single step followed by vigorous mixing. Lyophilisation was conducted on an α 1-4 LSC freeze-dryer (Martin Christ, Osterode, Germany).

The mean particle diameter (z-ave) and polydispersity index (PDI) were determined by DLS (Zetasizer NanoZS/ZEN3600, Malvern Instruments, Herrenberg, Germany). CryoSEM images were obtained on a JSM-7500F (Jeol, Tokyo, Japan) equipped with an ALTO-2500 liquid nitrogen (LN2) cryo-transfer system (Gatan Inc., Pleasanton, CA, USA). Samples were sputtered with platinum using the ALTO system. Optical microscope images were captured on a light microscope (Stemi 2000-C, Carl Zeiss, Jena, Germany) on samples prepared in transparent 96-well plates (Nunc, Thermo Fisher Scientific, Langenselbold, Germany).

Transfection efficiency *in vitro* was investigated via real-time-PCR (RT-PCR). SKOV3 cells were lysed 24 h after transfection. MRNA was isolated from culture cells and reverse transcribed to cDNA. Subsequently, RT-PCR was performed using a SYBR™ Green PCR Kit and a Rotor-Gene 3000 RT-PCR thermal cycler (Corbett Research, Sydney, Australia). Calibration curves for GAPDH and β-actin mRNA were prepared by serial dilutions of cDNA of the blank sample (untreated cells).

All measurements were carried out in triplicates and values are presented as mean values ± standard deviation.

z-ave and PDI of freshly prepared unloaded carriers were determined via DLS (95.2 ± 1.4 nm, PDI = 0.132 ± 0.019). Freeze-drying without lyoprotectant resulted in a fibrous polymer residue, forming large, visible aggregates upon rehydration. However, employing a c_{glc} of ≥1.5% led to incorporation of cariers into an amorphous, honey-like matrix and ensured virtually unchanged carrier size and size distribution after rehydration (Fig. 1A). CryoSEM images of freshly prepared nano-suspensions were in agreement with the results from light scattering experiments and revealed a uniform distribution with marginal aggregation. Images after rehydration proved that spherical carriers were still intact. However, despite unchanged carrier sizes as determined by DLS, onset aggregation to worm like assemblies was observed (Fig. 1C). Stabilisation of unloaded carriers has previously been reported for other core–shell type particulate carrier systems (Chan et al., 2009), required concentrations of sugar protectant are consistent with the literature (Packhaeuser et al., 2009).

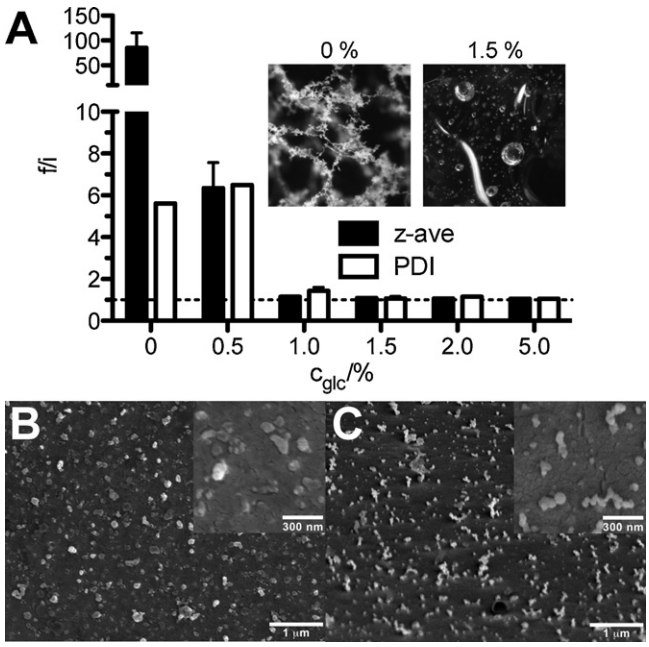

Fig. 1. Ratio of final and initial (*f*/*i*) z-ave and PDI of nano-carriers, lyophilised as a function of c_{glc}. Insets: optical microscope images of dry nano-carriers, lyophilised with 0 and 1.5% of glc (A). Cryo-SEM images of freshly prepared (B) and rehydrated (C) nano-carriers (c_{glc} = 1.5%).

In addition to lyophilisation of unloaded carriers, the feasibility of complexation with siRNA was investigated. Generally, complexation could be carried out after (L1) or before (L2) lyophilisation (Fig. 2A). On the one hand, lyophilisation of pre-formed complexes, for formulation of easy-to-use transfection agents sounds appealing. On the other hand, there is the risk of nucleic acid degradation (Kundu et al., 2011) or alteration in complex structure (Hahn et al., 2010) during lyophilisation and long-term storage. For both strategies, direct lyophilisation in well plates (Fig. 2A) enables straightforward rehydration by addition of cell culture medium (Reinisalo et al., 2006). Stability of unloaded suspensions is mainly due to electrostatic repulsion of PEI charges. Successive neutralisation upon addition of siRNA leads to poor stability at low N/P ratios. Therefore, at these conditions, there is also an increased risk of aggregation during freeze-drying and the applicable route of lyophilisation and formulation is ultimately a question of N/P and the amount of lyoprotectant. z-ave and PDI of complexes, formed via strategy L1 or L2 at two different N/P ratios (10 and 20) were compared to their freshly prepared counterparts (Fig. 2B). No significant changes between freshly prepared complexes and those assembled after lyophilisation (L1) were observed. By contrast, formulation route L2 resulted in pronounced aggregation, especially at low N/P. An increased need of lyoprotectant with larger amounts of nucleic acid was expected (Yu and Anchordoquy, 2009) and therefore lyophilisation of complexes (L2) was further investigated at elevated glc concentrations (1.5–15%, Table 1). In case of N/P = 20

Table 1
Ratio of final and initial (*f*/*i*) z-ave and PDI of complexes lyophilised (L2) at two different N/P ratios (10 and 20) as a function of c_{glc}.

c_{glc} (%)	N/P = 10		N/P = 20	
	z-ave (*f*/*i*)	PDI (*f*/*i*)	z-ave (*f*/*i*)	PDI (*f*/*i*)
1.5	34.49 ± 5.29	3.93 ± 1.89	1.54 ± 0.13	2.06 ± 0.30
3.0	33.49 ± 4.74	1.80 ± 0.34	1.21 ± 0.04	1.24 ± 0.12
5.0	30.44 ± 3.47	1.82 ± 0.91	1.22 ± 0.13	1.13 ± 0.14
10.0	16.92 ± 8.75	3.59 ± 1.55	1.06 ± 0.01	1.26 ± 0.11
15.0	4.47 ± 1.01	2.61 ± 0.38	1.00 ± 0.01	1.21 ± 0.14

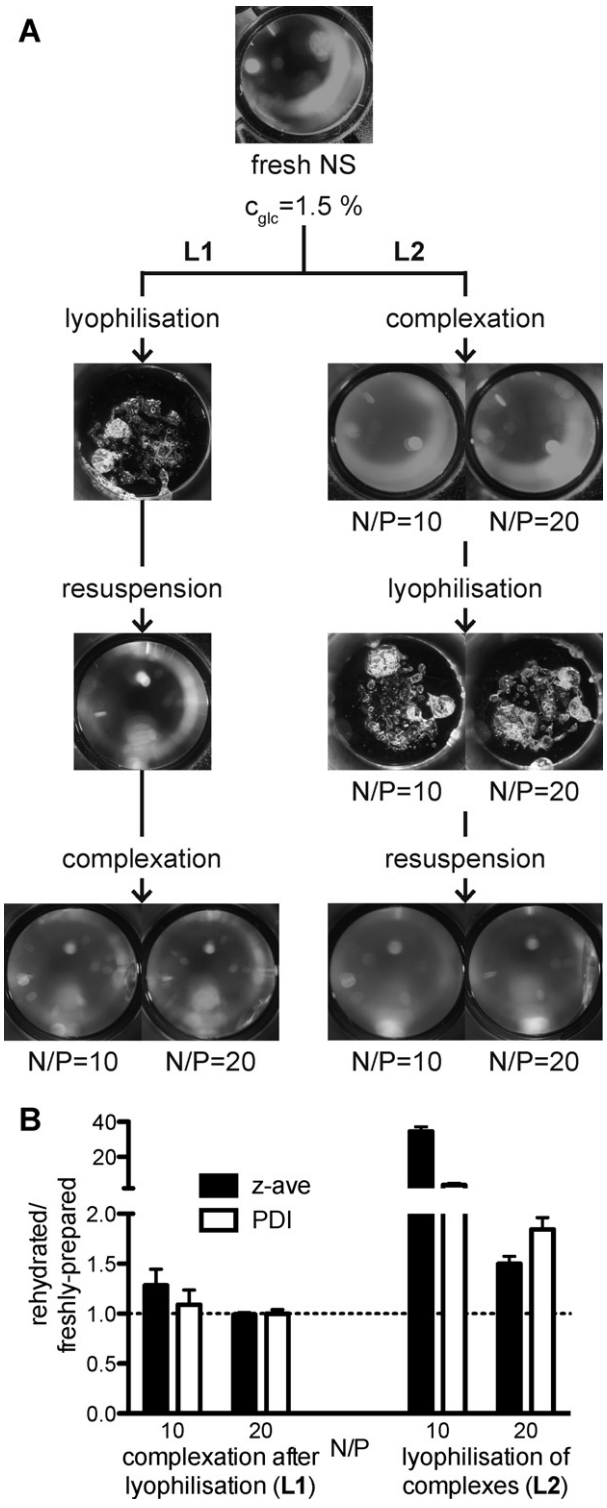

Fig. 2. Organisation chart of different formulation strategies (L1: complexation after lyophilisation, L2: lyophilisation of complexes). Nano-suspensions and residues after lyophilisation were captured with a light microscope in 96-well plates (A). Ratio of final and initial (*f*/*i*) z-ave and PDI of carriers loaded at various N/P ratios (10 and 20) and lyophilised by different strategies (L1, L2, c_{glc} = 1.5%) (B).

recovery of complex diameter was virtually attained above ~5% of glc. At N/P = 10 complete recovery of complex characteristics could not be achieved even at c_{glc} as high as 15%. Albeit there was a decrease in aggregation as a function of c_{glc} and carriers are likely to be regained by a further increase in protectant concentration, this would not be reasonable in order to attain highly concentrated, nearly isotonic nano-suspensions after rehydration.

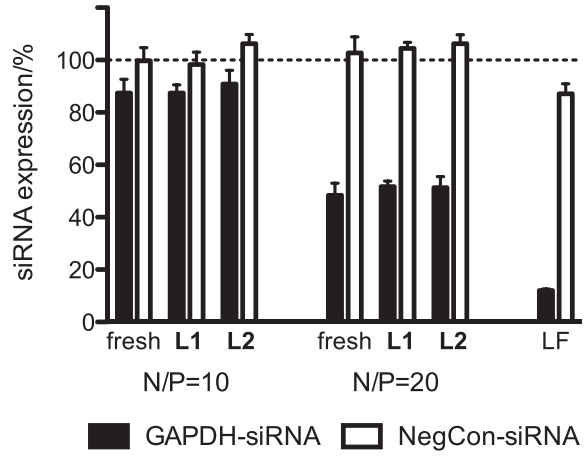

Fig. 3. Transfection efficiency (SKOV3-cells) was determined *in vitro* by RT-PCR. Hs_GAPDH-primers were used to quantify hGAPDH_gene expression, Hs_β-actin-primers were utilised as internal standard to determine relative expression levels for each gene. Lipofectamine (LF) was used as a positive control. Freshly prepared complexes were compared to ones assembled after (L1) and before (L2) lyophilisation (c_{glc} = 5%, N/P = 10 and 20).

Transfection activity of formulations, lyophilised via both strategies, was determined via RT-PCR and compared to freshly prepared complex suspensions (Fig. 3). Effective knock-down was detected at N/P = 20 and all stored formulations were as efficient as freshly prepared. This is in agreement with reports on lyophilised nonviral delivery systems (Andersen et al., 2008; Kundu et al., 2011), even though there is some controversy in the literature (Brissault et al., 2006). Therefore, minor changes in aggregation tendency of resuspended unloaded carriers (as previously observed in cryoSEM) are not regarded to be critical.

In conclusion, the results of this study demonstrate that lyophilisation is a very effective technique to produce physically stable PEG-PCL-PEI formulations. Size and PDI of unloaded nano-suspensions (95.2 ± 1.4 nm, PDI = 0.132 ± 0.019) were preserved during lyophilisation upon addition of lyoprotectant (~1.5% of glc). No significant difference between freshly prepared complexes and those assembled from lyophilised carriers was observed. Furthermore, higher glc concentration (~5%) facilitates the stabilisation of pre-formed carrier/siRNA complexes at elevated N/P ratio of 20. In case of lower N/P (10), stabilisation could not be achieved in the range of applicable c_{glc}. RT-PCR experiments *in vitro* proved, that all lyophilised formulations were as active as freshly prepared ones. These findings are fundamental and may be applicable to further charged particulate gene delivery systems to reproducibly shelf ready-to-use formulations for prospect *in vitro* and *in vivo* studies.

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