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Research paper

# Large-scale production of lipoplexes with long shelf-life

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**Abstract**

The instability of lipoplex formulations is a major obstacle to overcome before their commercial application in gene therapy. In this study, a continuous mixing technique for the large-scale preparation of lipoplexes followed by lyophilisation for increased stability and shelf-life has been developed. Lipoplexes were analysed for transfection efficiency and cytotoxicity in human aorta smooth muscle cells (HASMC) and a rat smooth muscle cell line (A-10 SMC). Homogeneity of lipid/DNA-products was investigated by photon correlation spectroscopy (PCS) and cryotransmission electron microscopy (cryo-TEM). Studies have been undertaken with DAC-30®, a composition of 3β-[*N*-(*N*,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol (DAC-Chol) and dioleylphosphatidylethanolamine (DOPE) and a green fluorescent protein (GFP) expressing marker plasmid. A continuous mixing technique was compared to the small-scale preparation of lipoplexes by pipetting. Individual steps of the continuous mixing process were evaluated in order to optimise the manufacturing technique: lipid/plasmid ratio, composition of transfection medium, pre-treatment of the lipid, size of the mixing device, mixing procedure and the influence of the lyophilisation process.

It could be shown that the method developed for production of lipoplexes on a large scale under sterile conditions led to lipoplexes with good transfection efficiencies combined with low cytotoxicity, improved characteristics and long shelf-life.


*Keywords:* Scale-up; Lipoplex; Non-viral gene transfer; Cytotoxicity; DAC-30®

## 1. Introduction

The potential to deliver DNA to cells for the purpose of gene therapy represents an enormous advance in the treatment of both inherited and acquired genetic disorders. Viral and non-viral vector systems, such as cationic lipids and polymers, have been widely investigated as possible delivery systems [1–3]. Viral vectors deliver genetic material more efficiently to cells than non-viral vectors [4]. However, they raise severe health problems like immunogenicity and toxic reactions [5–7]. In addition, purification and production on an industrial scale is a major obstacle. Whereas cationic lipids have been proven to be safe in clinical trials [8]. A limiting factor for their clinical practicability is the requirement of being freshly prepared prior to administration because of their tendency to aggregate and fuse in liquid formulations [9–15]. Numerous studies have demonstrated a number of parameters influencing lipoplex stability and quality [16–21]. This includes problems concerning control over the reproducible formation of the lipid/DNA complexes, inadequate handling by physicians and therefore resulting insufficient quality control and transfection efficiency of the gene transfer system. Thus, production of stable and easy to handle single vial lipoplex formulations is a worthwhile objective.

Efforts have been made in the past to develop automated production processes for lipoplexes [22]. However, this

*Abbreviations:* A-10 SMC, rat aorta smooth muscle cells; DAC-Chol, 3β-[*N*-(*N*,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol; DOPE, dioleoylphosphatidylethanolamine; EGFP, enhanced green fluorescent protein; GMP, good manufacturing practice; HASMC, human aorta smooth muscle cells; PCS, photon correlation spectroscopy; RT, room temperature; SMGS, smooth muscle growth supplement; TM, transfection medium; WFI, water for injection.

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method is restricted to small production volumes while the necessity of storing the product at −70 °C limits the possibilities of handling. Storing of lipoplexes in liquid form led to a decrease in transfection efficiency [15]. Freeze-drying has been suggested as a useful method for stabilisation and storage of polyplexes [23]. In contrast, freeze-drying of cationic lipid/DNA-complexes was recently reported to produce large, irregularly shaped particles [24] with a loss in transfection activity [25], whereas spray-drying resulted in stable respirable dry powder systems for respiratory gene delivery [24].

No adequate method has been reported for the production of large-scale batches of lipoplexes with sufficient shelf-life to date. In this study we introduce a continuous mixing method for the manufacturing of lipid/DNA-complexes on a large scale followed by lyophilisation for the development of stable lipoplex formulation with reproducible characteristics and increased shelf-life. In former studies we have evaluated DAC-30®, which has been reported in the literature to be applied in gene transfer experiments [26–29], as a transfection reagent with the suitability for production of pharmaceutical products in respect to transfection efficiency, toxicity and GMP-conformity [30]. Biological activity and cytotoxicity of the produced lipoplex product was assayed in a cell model for the treatment of cardiovascular diseases (human aorta smooth muscle cells, HASMC, and a stable and well established rat smooth muscle cell line, A-10 SMC).

In this study process parameters like pre-treatment of the lipid, lipoplex preparation, presence of cryoprotection components and the influence of the lyophilisation process were evaluated. Furthermore, characteristics and homogeneity of the produced batches were analysed by cryotransmission electron microscopy (cryo-TEM) for visualisation of lipoplex structure and by photon correlation spectroscopy (PCS) for size determination and physical stability.

## 2. Materials and methods

### 2.1. Materials

3β-[*N*-(*N*,*N*′-Dimethylaminoethane)-carbamoyl]-cholesterol (DAC-Chol)/dioleoylphosphatidyl-ethanolamine (DOPE) 3:7 (w/w) (DAC-30®) was obtained from G.O.T. Therapeutics GmbH (Berlin, Germany). A-10 rat smooth muscle cells (A-10 SMC) were obtained from DSMZ (Braunschweig, Germany) and HASMC were purchased from Tebu (Frankfurt/Main, Germany). Medium 231 supplemented with smooth muscle growth supplement (SMGS) was obtained from Tebu (Frankfurt/Main, Germany). Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum (FCS), phosphate buffered saline (PBS) and other culture reagents were obtained from Biochrom (Berlin, Germany). The plasmid pAH7-EGFP [expressing enhanced green fluorescent protein (GFP)] and the components of transfection mediums 1 and 2 [TM1, a solution containing sucrose (250 mM) and sodium chloride (25 mM), and TM2 containing mannitol (220 mM), sucrose (29 mM) and sodium chloride (25 mM)] were obtained from Boehringer Ingelheim Pharma GmbH & Co. KG (Biberach/Riss, Germany).

### 2.2. Methods

#### 2.2.1. Lipoplex preparation

*2.2.1.1. Small-scale preparation of lipoplexes.* Enhanced green fluorescent plasmid pAH7-EGFP (denoted in this paper as 'plasmid') was mixed with DAC-30® in lipid/DNA ratios (w/w) of 4:1 and 5:1 according to the following protocol: lyophilised DAC-30® was redispersed in TM1 at a concentration of 1 mg/ml and incubated for 30 min at room temperature. Plasmid DNA and the respective amount of DAC-30®-dispersion were diluted separately in equal volumes of transfection medium (TM1 or TM2) to obtain the desired lipoplex amount in 1 ml of lipoplex preparation. Dilutions were combined discontinuously by pipetting the liposomes into the plasmid solution, gently mixing and incubating for at least 30 min at room temperature to allow the formation of lipoplexes.

*2.2.1.2. Large-scale preparation of lipoplexes and lyophilisation protocol.* Sterile lyophilised DAC-30® was dispersed with sterile TM1 or TM2 at a concentration of 1 mg/ml and incubated for 30 min. The manufacturing process is shown schematically in Fig. 1. For the preparation of extruded liposomes the dispersion was pumped once through an 800 nm polycarbonate membrane (Millipore Isopore, Schwalbach, Germany) using a peristaltic pump (Filtron, Karlstein, Germany). In order to obtain a lipid/DNA ratio (w/w) of 4:1 or 5:1, plasmid and lipid-dispersion (not extruded or extruded) were diluted separately in sterile TM1 or TM2 to a concentration of 50 μg/ml DNA and 200 or 250 μg/ml DAC-30®, respectively. To achieve simultaneous mixing of lipid and DNA at a constant flow rate both preparations were mixed via a Y-connector (3.0 or 5.0 mm diameter, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach/Riss, Germany) using a Hundt PM pump (Techno Pack Hundt GmbH, Karlsruhe, Germany). Lipoplex dispersion was collected in an appropriate reservoir. Lipoplex formation was characterised 30 min after mixing by size measurement. Lipoplex dispersions (1.5 ml) were transferred into 2 ml lyophilisation vials (Schott, Mainz, Germany). Vials were placed on the shelf of an alpha 2–4 Christ lyophiliser (Christ, Osterode, Germany). The freeze-drying process occurred with the following cycle: freezing of the samples at −50 °C for 3 h at 1000 mbar chamber pressure. Main drying took place at −20 °C for 42 h and 30 °C for 6 h; chamber pressure was reduced to 0.05 mbar. Cooling of samples at 5 °C, which was also the final storing temperature until rehydration,

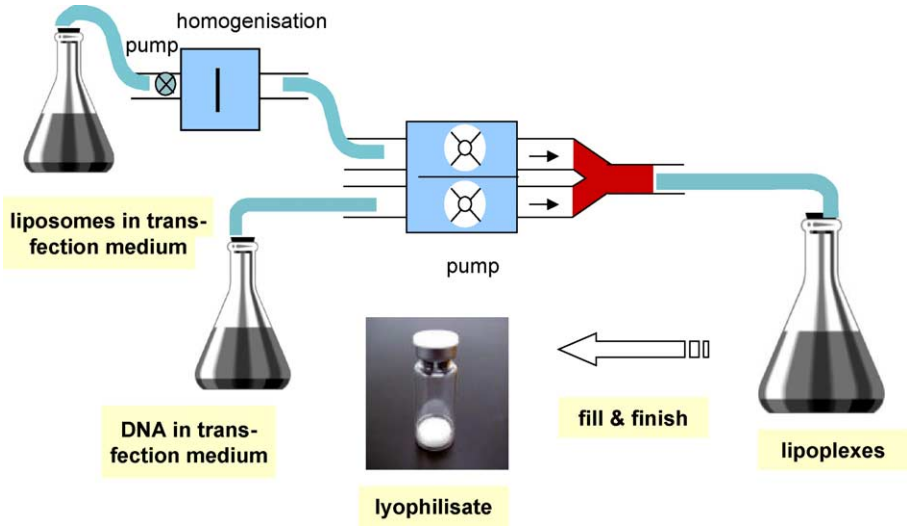



Fig. 1. Schematic representation of a large-scale manufacturing process. Lipid and plasmid, respectively, are diluted separately in transfection medium. Lipid is extruded once through a membrane with defined pore size (here 800 nm) and continuously mixed with equal volumes of plasmid in a second pump system via a Y-connector. The resulting lipoplexes are bottled and then lyophilised.

finished the cycle. Before removal, vials were capped under vacuum.

### 2.2.2. Cell culture

A-10 SMC were cultivated in DMEM supplemented with 20% (v/v) FCS and HASMC were maintained in Medium 231 with SMGS. Cells were cultivated at 37 °C in a humidified atmosphere with 5% (v/v) $CO_2$.

### 2.2.3. In vitro transfection assay

Cells were plated in a six-well cluster dish at a density of $7 \times 10^4$ cells 24 h prior to experiments and cultivated in the appropriate growth medium with serum. After 24 h in culture, the cells were washed with 2 ml PBS and 1 ml fresh growth medium containing serum was added to the cells.

Both freeze-dried and freshly prepared lipoplexes were studied. Freeze-dried lipoplexes were redispersed with 1.5 ml water for injection, incubated for at least 30 min at room temperature and diluted with transfection medium to the final concentration. One millilitre of freshly prepared or redispersed lipoplex-dispersions, respectively, was added to the cells. After incubation for 5 h at 37 °C (5% (v/v) $CO_2$) the supernatants were removed and 2 ml of the appropriate growth medium were added to each well. Thereafter, the cells were additionally cultured for a total of 48 h at 37 °C in 5% (v/v) $CO_2$.

### 2.2.4. Flow cytometric assay

Flow cytometric analysis of GFP fluorescence was performed using a four-colour FACS-Calibur (Becton Dickinson, Heidelberg, Germany) equipped with an argon laser exciting at a wavelength of 488 nm. For each sample, 10.000 events were collected by list-mode data that consisted of side scatter, forward scatter and fluorescence emission centred at 530 nm (FL1). The GFP fluorescence was collected on a logarithmic scale with 1024 channel resolution. Cell Quest Pro software (Becton Dickinson, Heidelberg, Germany) was applied for analyses. For sample preparation 48 h after transfection, cells were washed once with 2 ml PBS, collected by trypsinisation, pelleted, washed with 1 ml PBS and resuspended in 0.3 ml PBS.

### 2.2.5. Size of liposomes and lipoplexes

Size was measured by PCS using a Nicomp 380 (Nicomp, Santa Barbara, USA). Sample size was analysed by mono-modal Gaussian vesicle analysis with intensity weighting. Data represent the mean diameter obtained by three measurements.

### 2.2.6. Toxicity evaluation

CellTiter-Glo™ Luminescent Cell Viability Assay (Promega, Mannheim, Germany) was performed for determination of cell viability after transfection by quantification of the amount of ATP present, as produced by the metabolically active cells. Cells were transfected as previously described and toxicity evaluation occurred at the same point of time as the determination of transfection efficiency. Cell culture medium was replaced with 500 µl DMEM supplemented with 10% (v/v) FCS and equivalent amounts of test reagent. Contents of six-well plates were mixed for 2 min, incubated for 10 min at room temperature for cell lysis and transferred into 96-well plates. Three samples of 200 µl were taken out of the 1000 µl content of a single well of a six-well plate. A luminescent signal proportional to the amount of ATP present was generated. Quantification of the signal was carried out in a Luminometer (MicroLumat Plus LB 96 V, EG & G Berthold, Bad Wildbad, Germany). The percentage of cell viability was calculated by comparing the accordant luminescent signal to the signal obtained with non-transfected control cells.

*2.2.7. Cryotransmission electron microscopy*

A drop of the sample was put on a copper grid and coated with a holey carbon film. Most of the liquid was removed with blotting paper leaving a thin film stretched over the holes. The specimen was instantly shock-frozen by plunging into liquid ethane, cooled to 90 K by liquid nitrogen into a temperature-controlled freezing unit (Zeiss, Oberkochen, Germany). The remaining ethane was removed using blotting paper and the specimen was transferred into the electron microscope LEO 912 (Zeiss, Oberkochen, Germany) where micrographs were taken.

## 3. Results

In order to obtain a reproducible method for up-scaleable lipoplex products with improved shelf-life, a manufacturing method was developed and studied in many respects.

The following data were obtained with lipoplex products prepared on a large scale (Fig. 1) unless indicated otherwise.

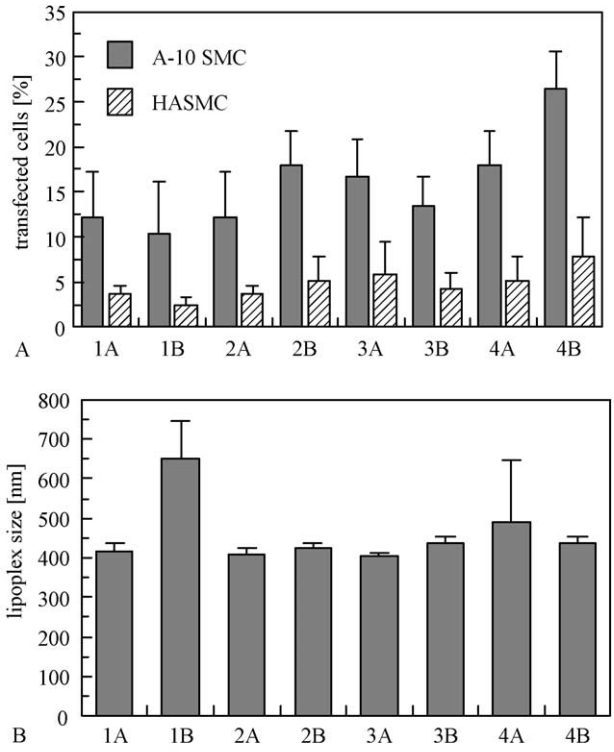
### *3.1. Transfection medium*

Two different transfection media containing cryo-protectants (TM1, TM2) were tested for optimisation of the lyophilisation process and lipoplex stability. Lipoplexes formed in TM1 containing 250 mM sucrose and 25 mM sodium chloride led to transfection results in both cell types comparable to those formed in TM2, which contained 220 mM mannitol, 29 mM sucrose and 25 mM sodium chloride (Fig. 2A). However, the different transfection media significantly affected complex size. Sizes of lipoplexes formed in TM2 went up to an average of 650 nm in comparison to an average size of 420 nm for lipoplexes formed in TM1 (Fig. 2B). Furthermore, the lipid/DNA formation of the lyophilised product in the presence of TM1 exhibited a better macroscopic structure. Therefore, TM1 was chosen as the favoured transfection medium.

### *3.2. Lipid/DNA ratio*

The most efficient lipid/DNA ratio (w/w) was evaluated in preliminary studies by analysing freshly prepared lipoplexes on a small scale (data not shown). The best transfection results were achieved with 4:1 and 5:1 (w/w) ratios. Consequently these ratios were applied in further studies. Lipid/DNA-products were manufactured on a larger scale with the continuous mixing method via Y-connector followed by lyophilisation.

The 5:1 (w/w) ratio appeared to be significantly more efficient in both cell types than the 4:1 (w/w) ratio (Fig. 2A). No influence on lipoplex size could be observed by variation




| sample | modified parameter | batch parameters | | | |
|---|---|---|---|---|---|
| | | TM | lipid/DNA ratio | diameter Y-connector | pre-treatment liposomes |
| 1A | TM | TM1 | 4:1 | 5 | not extruded |
| 1B | | TM2 | 4:1 | 5 | not extruded |
| 2A | lipid/DNA | TM1 | 4:1 | 5 | not extruded |
| 2B | ratio (w/w) | TM1 | 5:1 | 5 | not extruded |
| 3A | Y-connector | TM1 | 5:1 | 3 | extruded |
| 3B | | TM1 | 5:1 | 5 | extruded |
| 4A | pre-treatment | TM1 | 5:1 | 5 | not extruded |
| 4B | liposomes | TM1 | 5:1 | 5 | extruded |

Fig. 2. Influence of the variation of manufacturing parameters on (A) the transfection efficiency in A-10 SMC and HASMC and (B) the size of lyophilised lipoplex formulations. Analysed manufacturing parameters were transfection medium (TM1, TM2), lipid/plasmid ratio (4:1, 5:1 (w/w)), size of mixing device (3 and 5 mm) and lipid pre-treatment (not-extruded, extruded).

of the lipid/DNA ratio. The measured sizes for both lipoplex products were about 420 nm (Fig. 2B).

### *3.3. Y-connector*

Two different Y-connectors with diameters of either 3 or 5 mm were applied to analyse influence on mixing characteristics. Sizes of lipoplexes prepared with the two connectors were both in the range of about 420 nm, whereas transfection properties seemed to be slightly affected (Fig. 2A and B). The 3 mm device was used for further studies. However, for large preparation volumes the 5 mm device is more appropriate.

### 3.4. Lipid extrusion

Lyophilised DAC-30® was redispersed with TM1 or TM2 and used either directly or extruded once through an 800 nm polycarbonate membrane. Generally, transfection appeared to be more efficient with lipoplexes formed with extruded liposomes.

It was remarkable, that homogeneity of lipoplex size was not necessarily related to pre-treatment of the liposomes (Fig. 2B).

### 3.5. Small-scale versus large-scale preparation

Transfection efficiency of lipoplexes prepared by pipetting in small scale was compared to the efficiency of continuously mixed lipoplex preparation on a large scale (Fig. 3). In A-10 SMC no difference in results with the varying lipoplex preparations could be detected. However, in HASMC, the transfection with lipoplexes prepared on a small scale was slightly more efficient. Significant differences were observed in size stability and in homogeneity of lipoplex sizes during the storage of the liquid lipoplex formulation at room temperature (Fig. 4). The size of lipoplexes prepared in small scale with not extruded liposomes increased up to 1600 nm after 24 h. Sizes of extruded liposomes and large-scale lipoplexes prepared with extruded liposomes exhibited steady values over this period of time.

### 3.6. Physical stability and biological activity of lyophilised lipoplexes

Lipoplexes prepared on a large scale showed no change in size within the first 48 h after preparation (data not shown). Size stability of the lyophilised product was also maintained during 18 months of storage (4–8 °C) (Fig. 5).

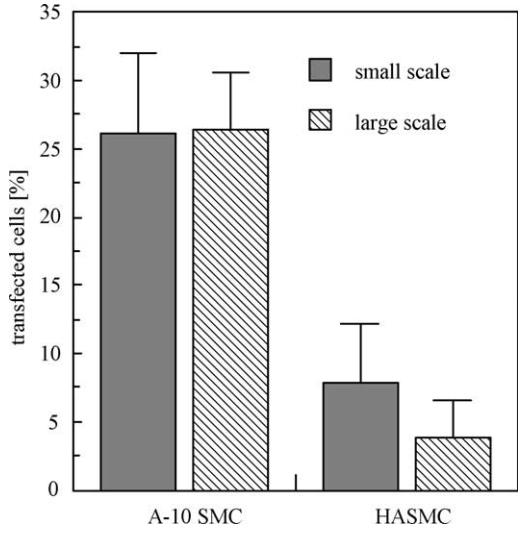



Fig. 3. Biological activity of lipoplexes produced in small and large scale in A-10 SMC and HASMC.

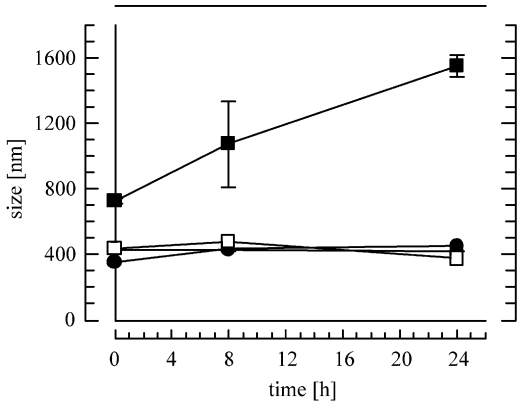



Fig. 4. Size progression of liposomes and lipoplexes within 24 h resting at room temperature after preparation: extruded liposomes (1×800 nm); lipoplexes prepared by continuous mixing with extruded liposomes before and after lyophilisation and lipoplexes prepared on a small scale with not extruded lipid.

An important aspect is the maintenance of biological activity of the lyophilised product. To date, the lyophilisate has been stored for 18 months at 4–8 °C and the transfection efficiency of the stored lyophilisates is comparable to the initial value obtained after preparation (Fig. 5).

### 3.7. Cell viability

The amount of cytotoxicity caused by transfection was measured 48 h after transfection as the percentage of non-viable cells relative to non-transfected control cells by quantification of the ATP amount produced by the remaining metabolically active cells.

With regard to the effect of pure plasmid and pure lipid on cell viability, the plasmid appeared to have no toxic influence. However, the lipid slightly affected the viability of both cell types and led to a decrease in the number of viable cells to about 80% of that in the control cells (data not shown). Generally, the presence of the lipoplexes was far

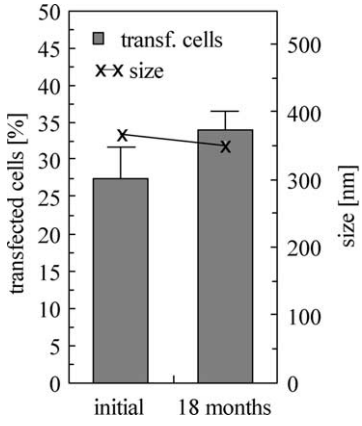
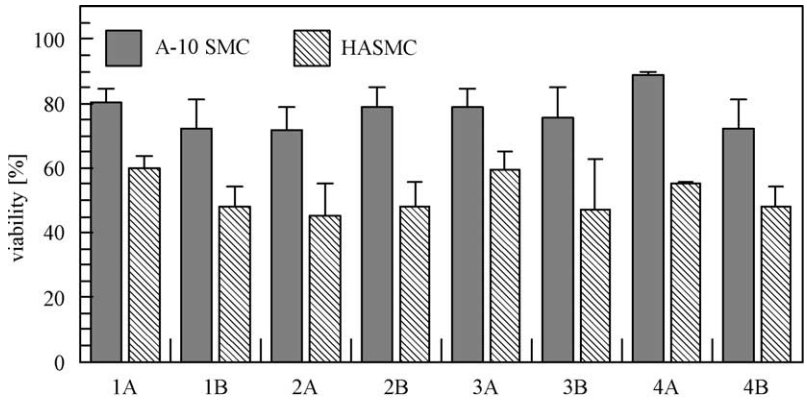



Fig. 5. Biological activity in A-10 SMC, indicated as bars, and size, indicated as line, of redispersed lipoplexes after preparation and after 18 months storage as lyophilisates at 4–8 °C.




| sample | analysed parameter | |
|---|---|---|
| 1A<br>1B | lyophilisation | before lyophilisation<br>after lyophilisation |
| 2A<br>2B | pre-treatment lipo-somes | not extruded<br>extruded |
| 3A<br>3B | lipoplex size (nm) | 370<br>1400 |
| 4A<br>4B | preparation technique | small scale<br>large scale |

Fig. 6. Cell viability of A-10 SMC and HASMC: influence of lyophilisation process, pre-treatment of liposomes, lipoplex size and preparation technique.

better tolerated by A-10 SMC than by the primary cells. As for efficiency experiments, the influence of several process parameters was analysed (Fig. 6). Viability values were in the range of 70–90% for A-10 SMC and 50–60% for HASMC. Toxicity was tested before and after the lyophilisation procedure, which turned out to exert only a slight decrease in cell viability. The analysis of lipoplexes containing either untreated lipid or lipid extruded once through an 800 nm membrane resulted in a slight increase in viability in A-10 SMC. Lipoplex size had no influence on A-10 SMC but led to a decrease of viability in HASMC. Cell viability of both cell types was reduced by transfection with lipoplexes produced in large scale.

In order to evaluate the effect of different concentrations of lipoplexes with a constant lipid/DNA ratio, the cell viability was evaluated after application of increasing amounts of a lyophilised lipoplex batch (Fig. 7). A decrease of viability in HASMC was detectable, whereas in A-10 SMC about the same level of viable cells was observed with the pure lipid and the lipoplexes.

### *3.8. Morphology of liposomes and lipoplexes*

For visualisation of particle structures from extruded DAC-30® liposomes, lipoplexes prepared on a small scale containing extruded liposomes, as well as lipoplexes prepared by continuous mixing after extrusion of liposomes were investigated by cryo-TEM (Fig. 8).

Cryo-TEM measurement of the pure liposome suspension extruded once through an 800 nm membrane without DNA revealed a large population of bilamellar liposomal vesicles (Fig. 8A). The difference in lipoplex preparation technique (small scale in Fig. 8B versus large scale in Fig. 8C) did not influence the morphology of the lipoplexes. Both preparations showed the same pattern.

## 4. Discussion

The aim of this study was to develop an up-scaleable single-vial formulation of DAC-30®/DNA lipoplexes with high physical stability and consistent biological activity.

Typically, the development of non-viral gene transfer systems focuses primarily on improving the transfection efficiency in various cell types. Often the physical stability

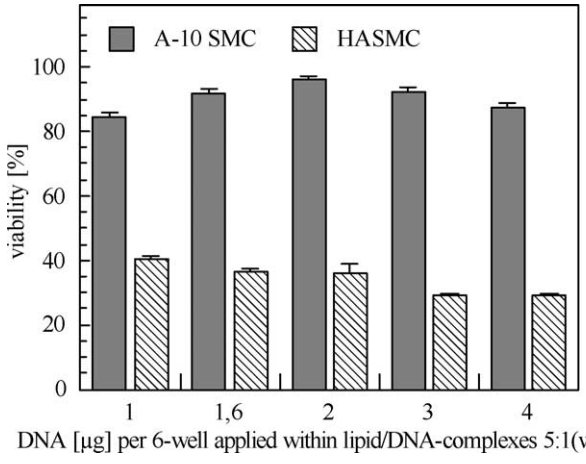



Fig. 7. Influence of viability on A-10 SMC and HASMC by increasing amounts of lipoplex product, lipid/DNA ratio 5:1 (w/w).

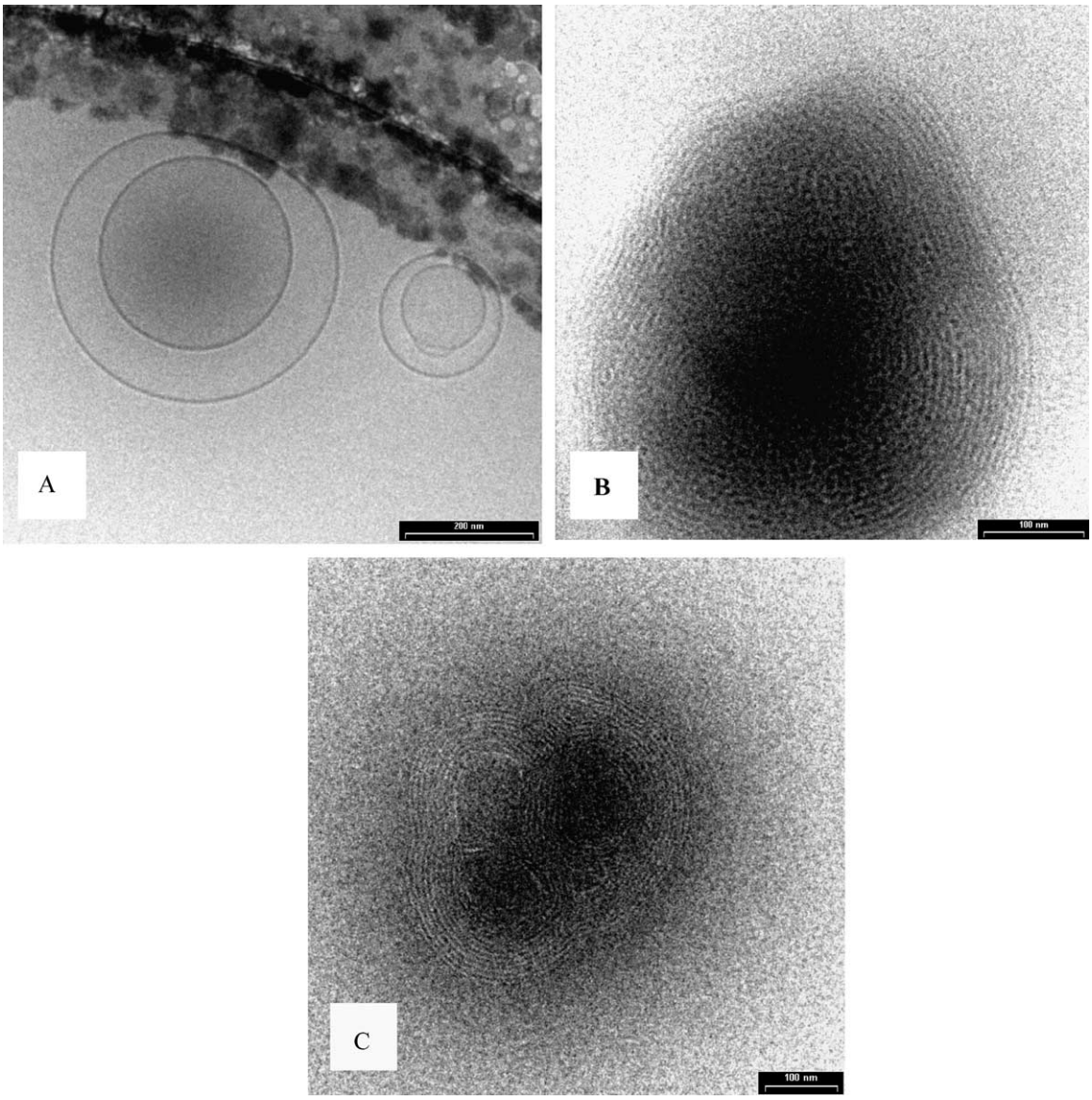



Fig. 8. Electron micrographs of different DAC-30® preparations after cryofixation (A) DAC-30® liposomes, extruded 1×800 nm, $c^{®}_{DAC\text{-}30}$=2,5 mg/ml. Bar represents 200 nm. (B) DAC-30® lipoplexes prepared on a small scale, lipid/DNA ratio 5:1 (w/w), $c^{®}_{DAC\text{-}30}$=0.25 mg/ml. Bar represents 100 nm. (C) Lyophilised and redispersed DAC-30® lipoplexes prepared on a large scale, lipid/DNA ratio 5:1 (w/w), $c^{®}_{DAC\text{-}30}$=0.25 mg/ml. Bar represents 100 nm.

of the applied systems is disregarded. Studies of Anchordoquy et al. [31] showed that the physical stability of non-viral formulations for gene delivery does not satisfy the requirements of approved pharmaceuticals. To obtain stable, high quality formulations with reproducible sizes it is essential to develop gene delivery systems and formulations with constant physical and chemical properties suitable for large-scale production and with long-term shelf-life. To this purpose a continuous mixing method appropriate for large-scale production followed by lyophilisation was established. According to Zelphati et al. [22] equal volumes of liposome dispersion and DNA-solution were mixed at the same rate. In this study, lipid and plasmid dilutions were combined via pumping instead of through the use of syringes, which is limiting to the preparation volume. This allowed preparation of almost unlimited batch volumes. Liposomes extruded once through an 800 nm membrane were mixed with plasmid DNA via a Y-connector using a peristaltic pump. With this method equivalent amounts of lipid and plasmid were continuously combined and the influence of the sequence of addition of liposomes and DNA during complex formation observed in our studies (data not shown) as well as those reported by Zelphati et al. [22] could be eliminated. The established preparation process was performed under aseptic conditions and a sterile lipid/DNA-product was obtained.

Lipid/DNA-product preparations were optimised in several ways. A suitable transfection medium was evaluated. The combination of 250 mM sucrose and 25 mM sodium chloride turned out to be the most convenient

formulation. The lipid/DNA ratio of 5:1 (w/w) was found to transfect both, A-10 SMC and HASMC, efficiently and was therefore chosen as a standard ratio for the lyophilised batches. The use of liposomes, which were extruded once through an 800 nm polycarbonate membrane further improved lipoplex stability: lipoplex size was slightly reduced and homogeneity was essentially increased compared to lipoplexes prepared of not extruded liposomes. Transfection with redispersed lipoplexes was equal or only slightly less efficient than with lipoplexes prepared on a small scale. The size of the Y-connector did not affect the lipoplex characteristics as assayed by analysing the transfection efficiency, particle size or cytotoxicity.

Lyophilisation of the lipoplexes prepared by continuous mixing did not lead to a decline in lipoplex stability. There have been reports that alterations of lipoplex size before lyophilisation and after redispersion occur, which depend on the lipoplex formulation and lyophilisation parameters [32] but this could not be confirmed in our study. Lyophilised formulations retained physical characteristics as well as biological activity. An enormous advance of lyophilised preparations is their insensitivity with regard to agitation or temperature fluctuations during transport and storage in contrast to frozen or liquid formulations. It is an important commercial aspect that the lyophilised formulations can be stored at 4–8 °C over a time period of at least 1.5 years and easily redispersed when needed.

The batches produced were also evaluated for their influence on cytotoxicity. It could be shown that transfection was in general consistently toxic for the individual cell types. High cell viability could be observed in A-10 SMC, whereas for HASMC the cell viability was reduced. Different parameters of the manufacturing process like pre-treatment of the lipid and lyophilisation did not significantly influence the lipoplex characteristics and the cell viability. Measured toxicity was therefore not attributed to the manufacturing process but more likely to the sensitivity of the individual cell types to transfection.

By performing cryo-TEM, multilayer lipoplex structures according to earlier findings [10,33–35] were observed. Both small scale and large-scale lipoplex preparations led to the fingerprint like patterns of alternating lipid and DNA. The structures showed a high sensitivity to the electron beam, suggesting high material density of the vesicles caused by the presence of DNA [33].

Thus, the lipoplexes not only retain their biological active structure in the scale-up process but also the observed structures can be reproducibly found in the different batches produced. Moreover, the homogeneous particle size distribution obtained with PCS measurement was confirmed by cryo-TEM.

In this study a single vial lipoplex formulation with appropriate transfection efficiency, combined with acceptable cytotoxicity and reproducible lipoplex size and structure was developed. Maintenance of biological activity was verified during 18 months of storage at 4–8 °C. The method offers the possibility for the production of gene transfer systems under sterile conditions on a large scale with increased shelf-life and appropriate storage conditions.

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