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Note

The establishment of an up-scaled micro-mixer method allows the standardized and reproducible preparation of well-defined plasmid/LPEI polyplexes

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ABSTRACT

Polyplexes based on linear polyethylenimine (LPEI) and plasmid DNA are known as efficient non-viral gene delivery systems. However, the requirement for freshly prepared complexes prior to administration due to their instability in aqueous suspension poses the risk of batch-to-batch variations. Therefore, the aim of the study was the establishment of a reproducible and up-scalable method for the preparation of well-defined polyplexes.

Polyplexes consisting of pCMVLuc plasmid and 22 kDa linear polyethylenimine (LPEI) were prepared by classical pipetting or with a micro-mixer method using different mixing speeds and plasmid DNA concentrations (20–400 μg/mL). The *z*-average diameter of the polyplexes was measured by dynamic light scattering. Metabolic activity and transfection efficiency was evaluated on murine neuroblastoma cells after transfection with polyplexes.

When varying mixing speeds of the micro-mixer, polyplex size (59–197 nm) and polydispersity index (0.05–0.19) could be directly controlled. The *z*-average diameter (65–170 nm) and polydispersity index (0.05–0.22) of the polyplexes increased with increasing plasmid DNA concentration (20–400 μg/mL).

The established up-scaled micro-mixer method allows the standardized and reproducible preparation of well-defined, transfection-competent plasmid/LPEI polyplexes with high reproducibility.

1. Introduction

Non-viral, plasmid-based therapeutics represent a new class of pharmaceuticals that offer the potential to cure several diseases currently considered untreatable [1]. Non-viral vectors, based on DNA complexes with cationic lipids (lipoplexes) or polycationic polymers (polyplexes), are attractive because of their low cost and high flexibility and have been proven safe and non-immunogenic in clinical trials [1,2]. On the other hand, depending on the polymer used, polyplexes suffer from drawbacks such as toxicity and non-specific interactions with the cells [3]. Another limiting factor for their clinical practicability is the requirement for freshly prepared formulations prior to administration because of the tendency towards particle aggregation in liquid formulations [2,4]. However, the day-to-day preparation poses the risk of batch-to-batch variability, including significant variations in product quality, safety and transfection rates, and the inability to perform extensive quality control prior to the actual administration due to time constraints [2,4]. Currently, the classical preparation of non-viral vectors, mixing a solution of DNA with a solution of cationic agents (i.e. lipid or polymer) via pipetting, is restricted to relatively small volumes [1]. This poorly defined and difficult to control preparation method can result in the formation of highly heterogeneous complexes with a broad range of sizes and charge ratios [5]. Thus, the controlled reproducible production of standardized batches of well-defined non-viral complexes is a major challenge [4,5]. Zelphati et al. [5] developed an automated and controllable production process for the preparation of lipoplexes utilizing a specially designed continuous flow pumping system, in which the DNA and liposome solution are mixed at 90° angles at the junction of a T-connector. In accordance to that first effort, Clement et al. [4] introduced a continuous mixing technique for the large-scale preparation of lipoplexes. However, such automated up-scaled mixing has not yet been established for the preparation of polyplexes.

Hence, we aimed at establishing an up-scaled method for the reproducible preparation of well-defined polyplexes based on plasmid DNA and 22 kDa LPEI. The influence of the preparation procedure (classical pipetting versus up-scaled preparation), the mixing speed and the plasmid/LPEI concentration on particle size, polydispersity and *in vitro* performance (transfection efficiency and toxicity) of the polyplexes was investigated.

Abbreviations: LPEI, 22 kDa linear polyethylenimine.

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2. Materials and methods

2.1. Materials

The plasmid (pCMVLuc) was produced by PlasmidFactory (Bielefeld, Germany). As described, 22 kDa LPEI was synthesized by acid-catalyzed hydrolyses from commercial poly(2-ethyl-2-oxazoline) (Sigma Aldrich, Steinheim, Germany) [6]. Plasmid and LPEI stock solutions were diluted in 10 mM L-histidine (Merck, Darmstadt, Germany) buffer pH 6.0, so that mixing equal volumes of the two dilutions always resulted in a N/P ratio (molar ratio of LPEI nitrogen (N) to DNA phosphate (P)) of 6/1. Indicated polyplex concentrations always refer to the plasmid DNA concentration of the samples.

2.2. Preparation of plasmid/LPEI polyplexes by pipetting

For the traditional preparation of the polyplexes ($n = 3$), 250 µl of plasmid solution were pipetted into the same volume of LPEI solution in a 1.5-mL reaction tube, mixed by rapid pipetting and incubated for 30 min at room temperature. For direct comparison with the up-scaled method, polyplexes were also prepared ($n = 3$) at increased volumes, mixing 2.5 ml of plasmid solution with 2.5 ml of LPEI solution with a 5-mL pipette in a 15-ml reaction tube.

2.3. Up-scaled preparation of plasmid/LPEI polyplexes

Equal volumes (2.5 mL) of plasmid solution and LPEI solution were loaded into two separate syringes with luer lock tip (Terumo, Leuven, Belgium), connected via polyetheretherketone tubings (0.5 mm inner diameter, 5.0 cm length) to a T-connector (Micro Tee P-890) (Upchurch Scientific, Oak Harbor, USA) and fixed into two common syringe drivers. The mixing occurred when the carriages on the syringe driver pushed the plunger of the syringes simultaneously at the same speed. The flow rate could be controlled either by using different-sized syringes (2.5, 5.0 or 10.0 mL) and/or by adjusting the plunger speed (0.1, 0.2, 0.5 or 1.0 cm/min) on the syringe driver. After mixing ($n = 3$), the polyplexes were incubated for 30 min at room temperature.

2.4. Particle size determination

The *z*-average particle diameter was measured by dynamic light scattering (DLS) using the DLS plate reader DynaPro Titan (Wyatt Technology, Dernbach, Germany) at a laser wavelength of 830 nm and a scattering angle of 150°. One hundred microliters per sample ($n = 3$) was pipetted into 96 UV-well plates (Costar™, Corning, USA) and analyzed at room temperature using five acquisitions, with 5 s each. Polyplexes were assumed as linear polymers with a refractive index increment value dn/dc of 0.185, and for the dispersant, the refractive index of water (1.33) was used. The functionality of the DLS plate reader was checked by polystyrene nanosphere-sized standards (Thermo Fisher Scientific, Fremont CA, USA). DLS autocorrelation data were analyzed with the Dynamics V6 software based on cumulant analysis with respect to particle size and polydispersity.

2.5. Cell culture

In vitro transfection experiments were performed using murine neuroblastoma (Neuro-2A) cells. Two parallel transfection series were carried out in separate 96-well plates. Cells were seeded 24 h prior to transfection with a density of 10^4 cells in 200 µL/well of culture medium containing 10% serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Immediately before transfection, medium was removed and 100 µL of polyplexes diluted in culture medium (200 ng DNA) was added to the cells for 24 h until cytotoxicity and luciferase activity were analyzed. Cytotoxicity was evaluated 24 h after treatment by methylthiazoletetrazolium (MTT)/thiazolyl blue assay [7]. Metabolic activity (%) was expressed relative to the metabolic activity of untreated control cells (HBG buffer pH 7.4 only (5% glucose, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (both, Merck, Darmstadt, Germany)), defined as 100%. To determine the luciferase reporter gene expression, the medium was removed and cells were lysed in 50 µl 0.5X Promega cell lysis solution 24 h after initial transfection. Luciferase light units were recorded with a Lumat LB9507 instrument (Berthold, Bad Wildbad, Germany) from a 22 µL aliquot of the cell lysate with 10-s integration time after automatic injection of freshly prepared luciferase assay reagent using the Luciferase assay system (Promega, Mannheim, Germany). Luciferase activity (%) of the samples was expressed relative to the luciferase activity of plasmid/LPEI control polyplexes (formulated in HBG buffer via pipetting), defined as 100%.

3. Results and discussion

3.1. Establishment of the up-scaled micro-mixer method for the preparation of plasmid/LPEI polyplexes

Particle size and aggregation behavior of the complexes are strongly affected by the preparation conditions like the way of adding the polymer solution to the nucleic acid solution or vice versa, diluting the complexes after their preparation or the used complexation time [8]. Up to date, the classical preparation of plasmid/LPEI polyplexes is to mix plasmid and LPEI solutions via pipetting that is traditionally performed at small volumes and

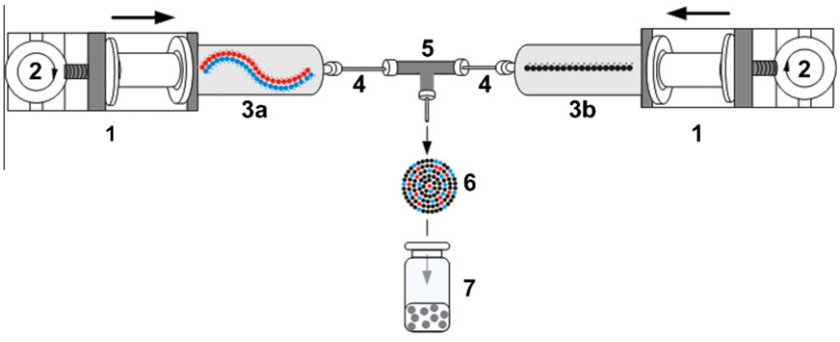

Fig. 1. Schematic representation of the up-scaled micro-mixer method for the preparation of plasmid/LPEI polyplexes: (1) syringe driver, (2) plunger speed control, (3a) syringe with luer lock tip loaded with poly-nucleic acid solution, (3b) syringe with luer lock tip loaded with polycation solution, (4) polyetheretherketone tubings, (5) T-connector site of mixing, (6) formed polyplex, (7) glass vial. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

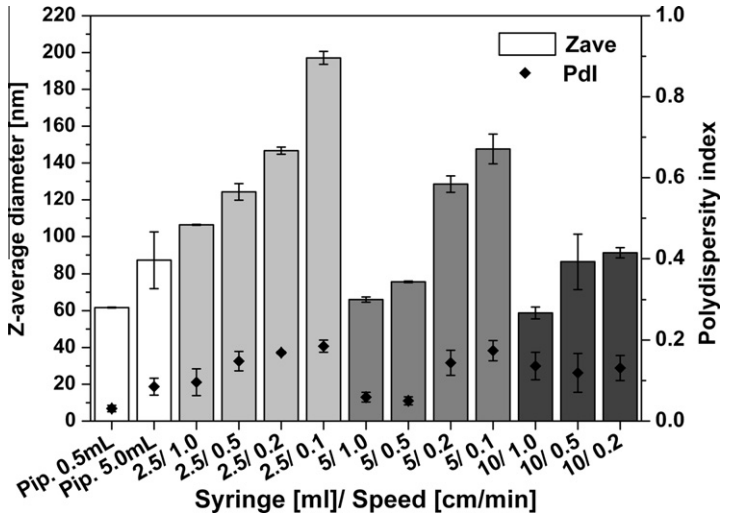

Fig. 2. *z*-Average diameter (nm) and PdI of plasmid/LPEI polyplexes (20 μg/ml) prepared by classical pipetting (Pip.) at low volumes (0.5 mL) or at large volumes (5 mL) or by the up-scaled preparation method using different syringe sizes (2.5, 5 and 10 ml) and different plunger speeds (1.0, 0.5, 0.2 and 0.1 cm/min). Values represent the mean ± 1 SD ($n = 3$).

often results in more or less heterogeneous batches [1,5]. Therefore, we established an up-scaled method for the preparation of polyplexes in accordance to the method described by Zelphati et al. [5]. Using two common syringe drivers, the plasmid and LPEI solutions were mixed at the junction of a T-connector and the mixing speed was controlled by syringe size and/or plunger speed (Fig. 1). Plasmid/LPEI polyplexes prepared by the up-scaled micro-mixer method at varied mixing speeds were compared to those prepared by classical pipetting. In general, with increasing mixing speeds, achieved by using increased plunger speeds and/or bigger syringe sizes, the *z*-average diameter as well as the polydispersity index (PdI) of the plasmid/LPEI polyplexes decreased from approximately 200 to 50 nm and 0.2 to 0.05, respectively (Fig. 2). Only when using a 10-mL syringe, the polydispersity index of about 0.15 remained constant with increasing flow rate as the handling of the small filling volume compared to the high volume of the syringe was hindered. In addition, polyplexes were prepared by pipetting at large volume (5 mL). This preparation method resulted in larger and more heterogeneous polyplexes compared to pipetting at low volumes (0.5 mL). As traditionally pipetting and using the up-scaled micro-mixer method with a mixing speed of about 10.6 mL/min (1.0 cm/min in combination with a 5-mL syringe, 13 mm inner syringe diameter) led to comparable results (polyplex size 65 nm, PdI 0.05), polyplexes prepared with these two settings were tested in cell culture experiments (Fig. 3). No differences in metabolic activity or reporter gene expression for up-scaled and classically prepared polyplexes were observed. Hence, we conclude that *in vitro* results were not significantly influenced by the preparation method.

Large batches of polyplexes should not be simply prepared by mixing increased volumes of plasmid and polymer solution via pipetting. This approach can lead to heterogeneous batches of larger polyplexes, as the mixing process at larger volume becomes less controllable. The establishment of a controllable and up-scalable micro-mixer method for the highly reproducible preparation of homogenous, standardized and well-defined polyplexes can minimize handling inconsistencies among different operators. Concerns regarding the order of addition, adding the DNA solution to the polymer solution or vice versa, are avoided by mixing the two solution feeds at a constant N/P ratio during the entire procedure leading to homogenous and standardized batches. Moreover, by using this method, the size and polydispersity of the plasmid/LPEI polyplexes can be directly controlled via the mixing speed. Up to now, an automated up-scaled mixing method was only established for the preparation of lipoplexes [4,5]. Zelphati et al. [5] introduced an analogous method for the preparation of cationic lipoplexes with well-defined sizes and also demonstrated that the particle size is influenced by the mixing rate. Using this method, the preparation volume is limited by the syringe size but can be easily scaled up. For example, Clement et al. [4] showed that mixing the two solutions via pumping is a viable alternative to the use of syringes, allowing the preparation of almost unlimited batch volumes.

3.2. Influence of the plasmid concentration on particle size and polydispersity of plasmid/LPEI polyplexes

In a next step, the influence of the preparation concentration on particle size and polydispersity of plasmid/LPEI polyplexes was evaluated. Plasmid/LPEI polyplexes were prepared at various plasmid DNA concentrations by the up-scaled micro-mixer method and compared to polyplexes prepared by classical pipetting (Fig. 4). The *z*-average diameter and the PdI decreased with decreasing

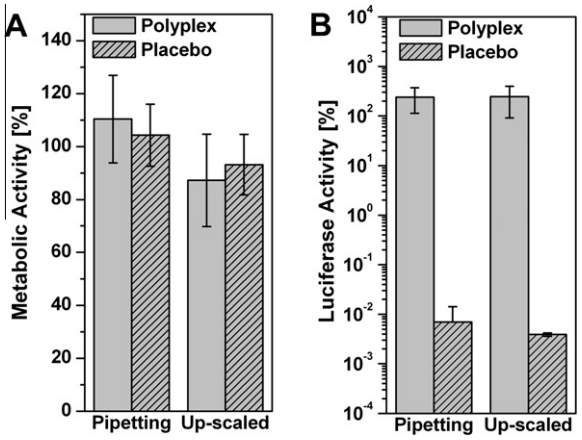

Fig. 3. Influence of the preparation method, pipetting (0.5 mL) versus up-scaled preparation, of freshly prepared plasmid/LPEI polyplexes and corresponding placebo formulations (only buffer) on (A) the metabolic activity (%) relative to HBG-treated murine neuroblastoma (Neuro-2A) cells determined via MTT assay and (B) *in vitro* transfection activity in murine neuroblastoma (Neuro-2A) cells determined as luciferase activity, normalized to the activity of the LPEI/CMVLuc control. Experiments were performed in quintuplicates using N/P 6.

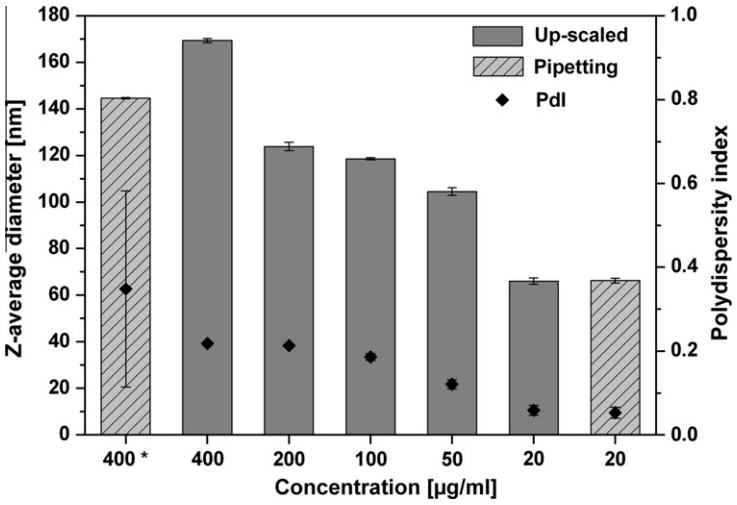

Fig. 4. *z*-Average diameter (nm) and PdI of plasmid/LPEI polyplexes prepared at different concentrations (20, 50, 100, 200 and 400 µg/ml) in L-histidine buffer pH 6.0 by classical pipetting (0.5 mL) and by the up-scaled preparation method using a 5-ml syringe and a mixing speed of 1.0 cm/min ($n = 3$. *Sample had to be diluted (1:10) prior to DLS measurement).

plasmid DNA concentration and ranged from 170 nm respectively PdI 0.22 at a plasmid DNA concentration of 400 µg/mL to 65 nm respectively PdI 0.05 at 20 µg/mL. At low concentrations, the *z*-average diameter and the PdI for polyplexes prepared by the up-scaled micro-mixer method were identical compared to those prepared by classical pipetting. However, with increasing concentration, the polydispersity of samples prepared by classical pipetting increased drastically compared to the micro-mixer method. The sample at 400 µg/mL prepared by classical pipetting had to be diluted for the dynamic light-scattering measurements due to high scattering by extremely large particles.

The results indicate that at higher concentrations, polyplexes become more unstable, leading to a stronger aggregation. This fact has been already observed and discussed in literature [9,10]. For example, Duguid et al. [10] showed that the hydrodynamic diameter of polyplexes composed of poly-(L-lysine) and synthetic polypeptides varied from 30–60 nm at a DNA concentration of 20 µg/mL to 80–160 nm at 400 µg/mL along with a large increase in polydispersity. Commonly, polyplexes are prepared at 50 µg/mL for *in vitro* experiments, whereas higher concentrations of 1–5 mg/mL can be only achieved if polyplexes are chemically modified to inhibit aggregation [11]. Especially at high concentrations, a more distinct heterogeneity of the polyplexes was observed when prepared via the classical preparation method. An explanation for this might be that higher polymer and DNA masses are mixed in a less controlled fashion at one time.

4. Conclusion

It is very difficult to achieve the same quality attributes of polyplexes when these carrier systems are prepared at large volumes by simple pipetting compared to the standard laboratory pipette mixing at low volume. The established micro-mixer method allows the highly reproducible preparation of large, standardized batches of homogenous, well-defined and transfection-competent polyplexes, banishes the risk of batch-to-batch variations and prevents handling inconsistencies among different operators, resulting in an increased polyplex quality. Moreover, by using this preparation method, the size and polydispersity of plasmid/LPEI polyplexes can be directly controlled via the mixing speed. The *z*-average diameter and the PdI of the polyplexes increased with increasing plasmid DNA concentration. The benefit of the up-scaled micro-mixer method is accentuated especially at high concentration as far less heterogeneity was observed in the up-scaled micro-mixer method compared to the classically produced particle preparations. Using this method, the batch volume can be easily further increased, which might be of high importance with regard to the production of large standardized batches of stable polyplex formulations. Thus, the possibility to reproducibly manufacture large standardized batches of well-defined, transfection-competent polyplexes is an important step closer from promising technology to clinical application.

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