Lab on a Chip

Cite this: *Lab Chip*, 2012, **12**, 2498–2506

www.rsc.org/loc

PAPER

Optimized preparation of pDNA/poly(ethylene imine) polyplexes using a microfluidic system

Heiko Debus, Moritz Beck-Broichsitter and Thomas Kissel*

Received 17th February 2012, Accepted 21st March 2012
DOI: 10.1039/c2lc40176b

Poly(ethylene imine) (PEI) is an established non-viral vector system for the delivery of various nucleic acids in gene therapy applications. Polyelectrolyte complexes between both compounds, so called polyplexes, are formed by electrostatic interactions of oppositely charged macromolecules and are thought to facilitate uptake into cells. Such complexes form spontaneously and on lab scale they are usually prepared by mixing solutions through pipetting. Hence, an optimized preparation procedure allowing the scale-up of well-defined polyplexes would be of general interest. We developed a new method for microfluidic polyplex preparation on a chip. The mixing behaviour within the microfluidic channels was evaluated. Polyplexes with PEI and plasmid DNA were prepared using this method, in comparison to the standard pipetting procedure. Sizes and polydispersity indices of these complexes were examined. The influence of various parameters on the polyplex characteristics and the suitability of this production procedure for other PEI-based complexes were also evaluated. It was shown that polyplexes could easily be prepared by microfluidics. The ratio of PEI to DNA was most important for the formation of small polyplexes, whereas other parameters had minor influence. The size of polyplexes prepared with this new method was observed to be relatively constant between 140 nm and 160 nm over a wide range of complex concentrations. In comparison, the size of polyplexes prepared by pipetting (approximately 90 nm to 160 nm) varied considerably. The versatility of this system was demonstrated with different (targeted) PEI-based vectors for the formation of complexes with pDNA and siRNA. In conclusion, polyplex preparation using microfluidics could be a promising alternative to the standard pipetting method due to its suitability for preparation of well-defined complexes with different compositions over a wide range of concentrations.

Introduction

Poly(ethylene imine) (PEI) is a well-known non-viral vector for the delivery of plasmid DNA (pDNA) and other nucleic acids.[1,2] PEI was modified for enhanced stability and transfection efficiency as well as reduced cytotoxicity for example by PEGylation and moreover, was coupled to ligands for targeted delivery to specific cells or tissues.[3]

Polymer complexes, also designated as polyplexes, are formed by electrostatic interaction between the negatively charged pDNA and the positively charged polymer (*i.e.* PEI). Usually, polyplexes are prepared by adding the polymer solution to the DNA solution under vigorously pipetting.[1] The complexes are then allowed to mature during a short incubation time. Unfortunately, this complex formation *via* standard pipetting method leads to poorly-defined particles, in contrast to well-defined particles, that one would expect to be generated in a nanoparticle production process.[4] Depending on the type of polymer (*e.g.* linear or branched PEI), as well as the molecular weight, the particle sizes of the polyplexes formed are more or less uniformly distributed. Furthermore, preparation by pipetting has been found to be restricted to a lower concentration of polyplexes in solution, which limits its potential for use in *in vitro* and *in vivo* experiments, and especially in clinical studies, where higher polyplex concentrations are necessary.[5]

In general, microfluidic mixing devices were designed to achieve a rapid mixing of two or more components.[6] The problem of mixing in microfluidic systems is the very small Reynolds number, meaning that laminar flow of the fluids can be observed and turbulent mixing almost doesn't occur.[7] Consequently, mixing mainly depends on molecular diffusion.[8] The mixing principles could be classified in two groups, passive and active mixing.[9] If an external energy is applied for mixing, the device is designated as an active micromixer. Looking at passive micromixers on the contrary, the microfluidic channels were specially designed to achieve increased contact time and area of the samples for enhanced diffusion.[6] Energy was only applied for the pumping of samples in this case.[8]

Philipps-Universität Marburg, Department of Pharmaceutics and Biopharmacy, Ketzerbach 63, 35032, Marburg, Germany. E-mail: kissel@staff.uni-marburg.de; Fax: +49 6421 28 27016; Tel: +49 6421 28 25881

Microfluidic devices have become more and more attractive for biomedical applications.[10] Recently, some methods were developed whereby the authors tried to overcome the disadvantages of polyplexes by standardization of their preparation. For example, Kasper *et al.* have shown a pumping method using a T-connector for scaled-up polyplex preparation.[11] Their study with linear PEI(22 kDa) was designed to optimize the polyplex preparation in larger quantities. Koh *et al.* described a hydrodynamic focusing method for the preparation of polyplexes[12] and lipoplexes.[13] Optimized electroporation through the use of a microfluidic device was reported by Wang *et al.*[14] These studies described the first automated approaches for the formation of polyplexes with DNA, but there is a necessity of further advances in polyplex preparation methods due to the fact that conventional lipoplex preparation by different investigators has been shown to result in very different complex characteristics.[15]

Hence, the objective of the present study was the development and optimization of a microfluidic preparation method for polyplexes with improved physical properties like particle size and polydispersity index (PdI). The hypothesis was that the use of such a method would be suitable for the formation of small polyplexes with a narrow size distribution. Additionally, we assumed that the preparation of polyplexes on a chip would be a versatile method, which could be applied to different polymers as well as different nucleic acids. To verify this assumption we performed the microfluidic preparation of polyplexes with various PEI-based (targeted) polymers and DNA, as well as siRNA.

Materials and methods

Materials

PEI (25 kDa) and PEI (5 kDa) were gifts from BASF (Ludwigshafen, Germany). Tf-PEI (5 kDa) was synthesized as described previously.[16] Plasmid DNA (pDNA) pCMV-Luc was purchased from PlasmidFactory (Bielefeld, Germany). Messenger RNA (RNActive®) was kindly provided by CureVac GmbH (Tübingen, Germany). 2′-*O*-Methylated 25/27mer dicer-substrate RNA (DsiRNA) targeting firefly luciferase (FLuc) was obtained from Integrated DNA Technologies (IDT, Leuven, Belgium). Other chemicals of analytical grade were used without further purification.

Lab-on-a-chip microfluidic system

A microfluidic system, which could be equipped with chips for different applications (Epigem Limited, Redcar, UK), was used for polyplex preparation. It consists of a baseboard equipped with microfluidic channels of 100 μm × 100 μm and several fittings for the attachment of chips and fluid connections. For the preparation of polyplexes, chips (Epigem Limited, Redcar, UK) with different architectures were used and fitted onto the baseboard (Fig. 1). The chips were available with different mixing length. The fluid connections were attached to two syringe pumps (World Precision Instruments Germany GmbH, Berlin, Germany), equipped with either a 500 μl syringe (Hamilton, Bonaduz, Switzerland) with an inner diameter of 2.546 mm, or with a 2 ml syringe (BD, Madrid, Spain) with an inner diameter of 8.54 mm. These syringes were connected to the tubing *via* a needle of 0.5 mm in diameter and 25 mm in length (BD, Drogheda, Ireland). Volumes and concentrations of the solutions were different for the individual experiments and are given with the description of the experimental design at a time.

Evaluation of the mixing behaviour inside the microfluidic chips

The behaviour of the fluids inside the microfluidic chips was evaluated using coloured solutions. Solutions of trypan blue (~7 mg ml^{-1}) and 5(6)-carboxyfluorescein (~1 mg ml^{-1}) were prepared and filtered prior use through a 1.2 μm filter. Two syringes were loaded, each containing one of these coloured solutions, and tubing was pre-filled with the solutions before starting the pumps. Both pumps were started simultaneously. Mixing behaviour of the fluids was observed under a Stemi 2000-C microscope (Zeiss, Oberkochen, Germany) and pictures were taken using a Canon PowerShot G10 digital camera (Canon, Krefeld, Germany).

Preparation of polyplexes by microfluidics

One syringe was loaded with a polymer solution and the other one with a nucleic acid solution of different concentrations diluted in 5% glucose solution unless otherwise stated. Tubing was pre-filled with the solutions before starting the pumps and they were operated with varying flow rates, given in the descriptions of the individual experiments. Both pumps were started simultaneously. Polyplex solutions were collected in seven fractions of 20 to 60 μl and particle sizes of fractions 4 to 7 were measured.

Preparation of polyplexes by standard pipetting method

A polymer stock solution (1 mg ml^{-1}) and the nucleic acid stock solution were diluted in 5% glucose to different concentrations given with the description of the individual experiment according to the intended polymer nitrogen to DNA phosphate (N : P) ratio. Subsequently, 20 μl of the polymer dilution was added to the same volume of the nucleic acid dilution and the solution was mixed by vigorous pipetting. Three replicates were prepared for each sample.

Particle size measurement by dynamic light scattering

Particle sizes and polydispersity indices (PdI) were determined using a Zetasizer NanoZS (Malvern Instruments, Herrenberg, Germany), equipped with a helium neon laser with an excitation wavelength of 633 nm. An ultra micro cell (Malvern Instruments, Herrenberg, Germany), which is suitable for the measurement of very low volume samples, was filled with 20 μl of the sample solution. The instrument was operated in the auto mode at 25 °C and refractive index (1.330) and viscosity (0.8872 cP) of water were employed for data analysis. Triplicate (preparation by pipetting) or quadruplicate (preparation by microfluidics) samples were measured. The results were analyzed using Dispersion Technology Software (Malvern Instruments, Herrenberg, Germany) and are expressed as the mean of the *Z*-average values in nm ± standard deviations.

Fractionation of PEI (25 kDa)

Fractionation of PEI was performed according to a protocol published by Bieber *et al.* with a few modifications.[17] In brief, 3 g of a Sephadex G 50 fine resin (GE Healthcare, Uppsala, Sweden) were pre-swollen for 1 h at 4 °C in 300 ml demineralized and filtered water. 50 mg PEI (25 kDa) were added to the suspension

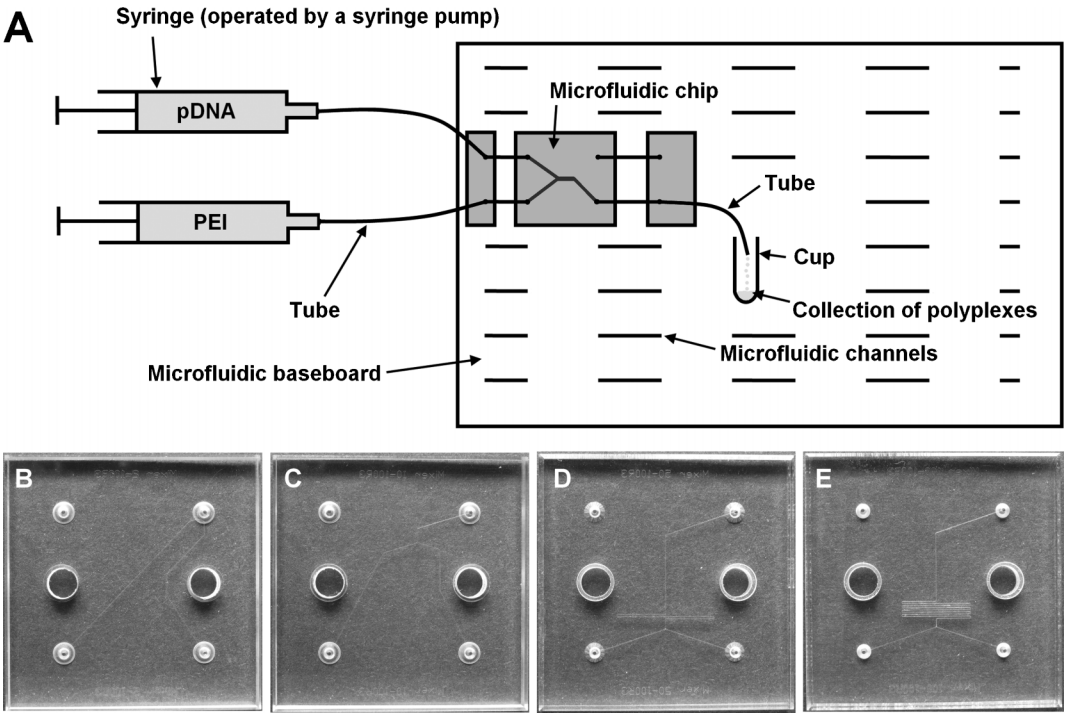

Fig. 1 (A) Schematic illustration of polyplex preparation by the microfluidic lab-on-a-chip assembly. Nucleic acid and polymer solutions were filled into a syringe and the liquids were pumped to the mixing chip, which was mounted on the microfluidic baseboard and connected to the syringes *via* tubing. Pictures of mixing chips (B) 2–100 R3 (C) 10–100 R3 (D) 50–100 R3 (E) 100–100 R3. The chips differ in lengths of the delay after the Y-connection of the fluids, which is given by the first number of the chip description in mm.

and the mixture was further incubated over night at 4 °C. A column (Millipore GmbH, Schwalbach, Germany) of 30 cm in length and a 1.7 cm inner diameter was filled with the suspension stepwise. A gel bed 16.5 cm in length resulted from this filling process. The column was rinsed for 3 h with a constant flow of 1 ml min^{-1} of water using an ÄKTA prime liquid chromatography system (Amersham Pharmacia Biotech, Uppsala, Sweden).

For the fractionation of PEI(25 kDa) a sample of 1 ml (50 mg ml^{-1}) was loaded onto the column and gel chromatography was performed with a constant water flow of 1 ml min^{-1}. Fractions of 1 ml were collected by an automated fraction collector (Amersham Pharmacia Biotech, Uppsala, Sweden), which was connected to the liquid chromatography system.

The fractions were analyzed in terms of PEI content *via* copper assay.[18] Briefly, samples of 20 µl per fraction were transferred to a 96 well plate (Sarstedt, Nümbrecht, Germany) and a 100 µl per well of a 0.02 M copper sulphate solution in 0.1 M potassium acetate was added. The plate was gently shaken at 400 rpm and room temperature for 1 min using a Thermomixer (HLC Biotech, Bovenden, Germany). Absorbance of the samples at a wavelength of 620 nm was determined using a FLUOStar Optima plate reader (BMG Labtech, Offenburg, Germany). Absorbance values were compared with a PEI standard curve (triplicate samples were measured for each concentration) and the PEI content of the pooled PEI (25 kDa) fractions was calculated using this standard curve.

Statistics

Results are shown as mean values ± standard deviations. Significance between means of two groups was tested using a student's *t*-test (Origin 7.0 SR0, Northampton, MA). A *p*-value < 0.05 was considered to be significant.

Results and discussion

A schematic illustration of the microfluidic lab on a chip system for polyplex preparation is given in Fig. 1A and pictures of the different mixing chips used in this study are shown in Fig. 1B–E. Table 1 gives an overview on different parameters influencing the formation of polyplexes.

Firstly, we placed the microfluidic baseboard with the assembled mixing chip under a microscope equipped with a digital camera in order to evaluate the fluid flow and mixing behaviour within the chips. The chips differ in length and design of the delay channel after the fluids are united in the Y-part of the chip. The length of this delay in mm is given by the first number of the chip designation. For the chips with 50 mm and 100 mm delay length the channel is arranged in loops (Fig. 1D–E). The syringes were filled with two differently coloured aqueous solutions, to allow for visualization of the mixing procedure. Fig. 2 shows the results of the experiments. It was clearly visible, that both fluids were flowing in parallel even after the Y-part where both fluids meet. This observation is in agreement with the general assumption of a laminar flow and almost no turbulence in microfluidic systems.[8] If a chip is not equipped with a loop after this Y-part (Fig. 2A), the parallel flow continued, even after the chip was passed completely. An initial mixing of the two fluids could be observed in the collecting cup, where the fluids merged slowly, presumably by diffusion. When pumping was stopped, diffusion of the liquids was observed inside the microfluidic channels as well.

Table 1 Variables in polyplex preparation. Parameters marked in bold were investigated in this study

Variables in polyplex preparation	
General	Microfluidic preparation
Individual concentrations of the solutions	**Individual flow rates of the solutions**
Concentration of polyplexes	**Total flow rate**
pH	**Chip design**
Solvent	
Ionic strength	

Of course, electrostatic interaction is the main driving force of polyplex formation. Nevertheless, an intensive perturbation of the two fluids may shorten the time necessary for the polyplex formation process to occur and may lead to a more reproducible process.

In contrast, when chips with additional loops after the Y-part were used, it was clearly observed that the fluids were mixed within these loops and a uniformly coloured solution left the chip (Fig. 2B and 2C). This observation was in accordance with the results of Fang *et al.*, showing improved mixing with an increasing number of nodes.[19] Generally, mixing in microfluidic systems depends on diffusion, and is enhanced by an increased contact time and area of the components.[6] This was achieved by the additional loops in the present system. Hence, it could be concluded, that chips with additional loops provided a more intensive mixing of the fluids than chips without loops.

Furthermore, the influence of different flow rates was investigated under the microscope as well. A difference in mixing behaviour could not be clearly observed, when the overall flow rate was changed between 10 μl min^{-1} and 50 μl min^{-1} (Fig. 2E). When different flow rates for both of the fluids were chosen, the fraction of the fluid with the lower flow rate was reduced in the parallel flow (Fig. 3E). Interestingly, the liquids still mixed during the loop passage to some extent, but the liquid leaving the chip was not uniformly coloured. Of course, these results do not give any information on the formation of polyplexes, because this depends mainly on their electrostatic interaction, but it could be concluded that an initial mixing of the polymer and DNA solutions would be necessary for the possibility of electrostatic interactions, and thus polyplex formation. Consequently, chips containing longer mixing channels with loops seemed more suitable for this application. Ho *et al.* reported a method for the monitoring of the assembly of DNA complexes with chitosan.[20] A method like this could be applied to PEI complex formation as well, if a kinetic evaluation of the polyplex formation is of interest.

In the following section of the study, the influence of different parameters (Table 1) on the preparation of PEI (25 kDa)/pDNA polyplexes with the microfluidic lab-on-a-chip system (Fig. 4) was evaluated. Different concentrations of both solutions as well as different flow rates were examined. Additionally, the overall flow rate and salt concentration were varied. The particle sizes of the polyplexes prepared seemed to depend mostly on the polymer nitrogen to DNA phosphate (N : P) ratio. The optimal N : P ratio to form small polyplexes was approximately between N : P = 5 and N : P = 20. In this range the polyplexes with smallest particle sizes and lowest PdI were formed. It seemed to be less relevant whether the concentration of the solutions or their flow rates were changed—there were of course differences in the exact polyplex sizes, however, the main influence was identified to be

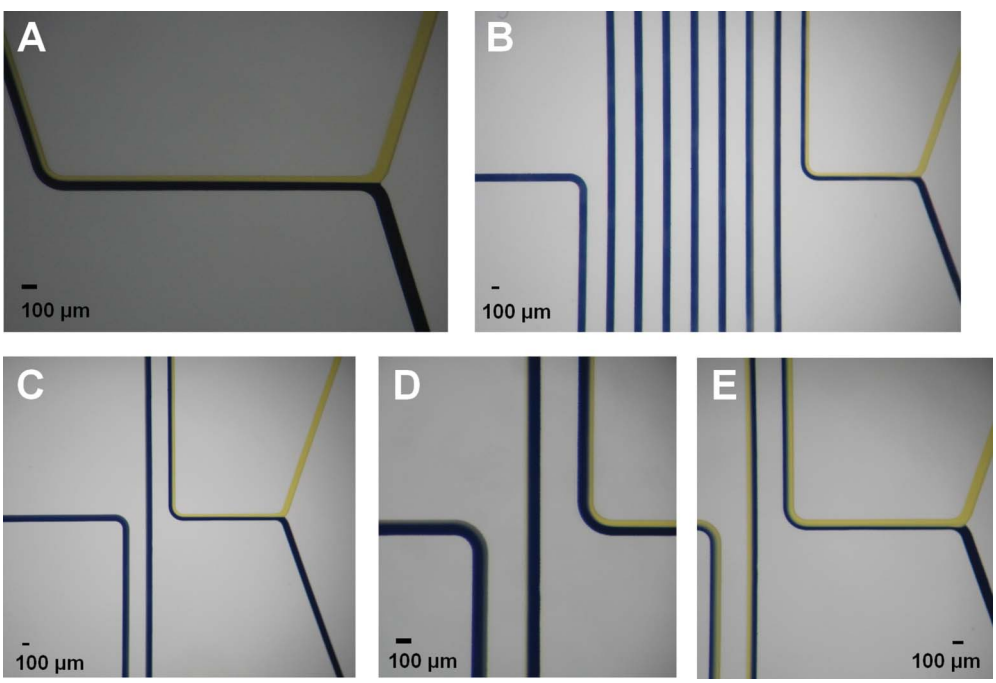

Fig. 2 Evaluation of mixing behaviour of aqueous liquids inside the microfluidic chips. Trypan blue and 5(6)-carboxyfluorescein solutions were pumped into the chip at a constant flow rate of 5 μl min^{-1} for both solutions. (A) Chip 10–100 R3 (B) Chip 100–100 R3 (C) Chip 50–100 R3 (D) Chip 50–100 R3 with a flow rate of 25 μl min^{-1} for both solutions (E) Chip 50–100 R3 with a flow rate of 25 μl min^{-1} for the yellow solution and 5 μl min^{-1} for the blue solution. Width of microfluidic channels (scale bar) is 100 μm.

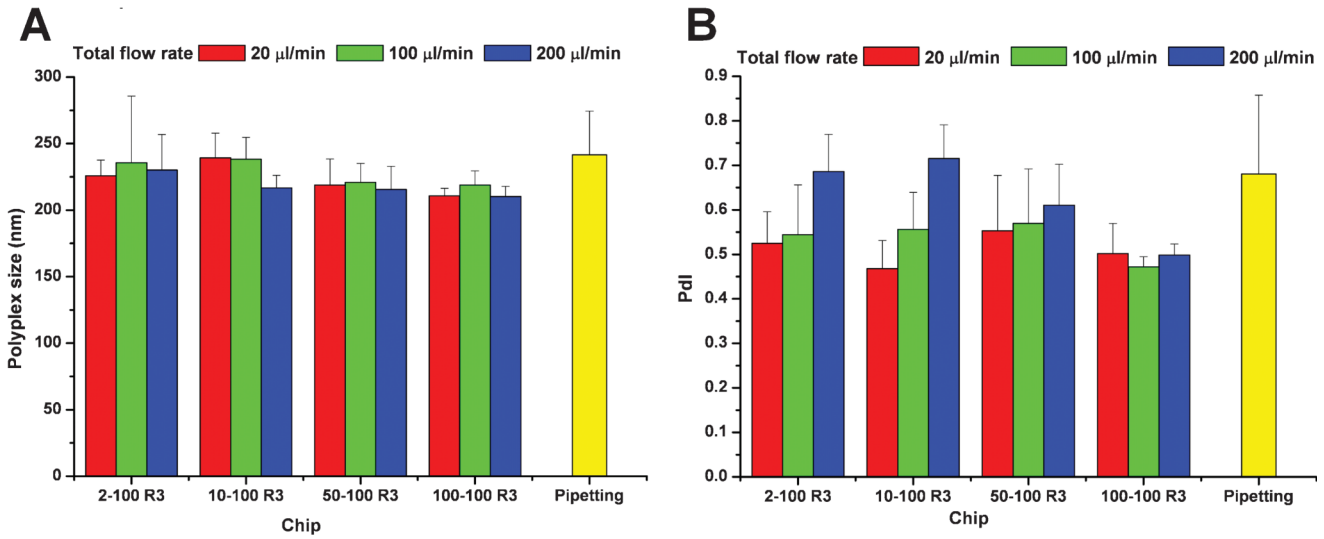

Fig. 3 Effect of chip design on (A) polyplex size and (B) polydispersity index of PEI–DNA polyplexes. For the microfluidic preparation, a constant flow rate of 50 µl min^{-1} was applied for both solutions (polymer nitrogen to DNA phospate ratio (N : P) = 10). Size and PdI were not significantly different in comparison to the polyplexes prepared by pipetting ($p < 0.05$).

the N : P ratio. For better comparison, the N : P ratio of 10 or near to 10 was marked with an arrow in Fig. 4A–D. This observation could be explained by the mechanism of polyplex formation, which depends mostly on electrostatic interactions. Other parameters seemed to be of minor impact for this process.

A confirmation of this assumption was possible by the analysis of the overall flow rate as well. While the ratio of one flow rate to the other was kept constant and the overall flow rate was increased, only slightly changing particle sizes were observed (Fig. 4E). This is also in agreement with the observations for coloured solutions (Fig. 2). It could be concluded, that the intensity of mixing is more important for the formation of polyplexes than the speed. In contrast, the PdI seemed to increase slightly at higher flow rates. Here, the speed obviously did have an influence and it took some time to form homogeneously distributed polyplexes.

The salt concentration of the solutions played a specific role on the particle sizes of the polyplexes.[21,22] This factor did influence the particle size to a high extent as well—it has to be taken into account that aggregation of non-shielded polyplexes will occur at higher salt or serum concentrations and they will be less stable over time.[23] This could be an explanation for the decreasing particle sizes at NaCl concentrations >150 mM, where larger particles are expected to sediment and therefore only smaller particles were measured.

After having determined the overall flow rate not to be critical to the particle sizes and the N : P ratio to be the most relevant parameter, more variables of the lab-on-a-chip production of polyplexes were of interest. Since different mixing chips are commercially available for the microfluidic baseboard and deviations of the mixing process in these chips were observed using coloured solutions (Fig. 2), the effect of these different chip architectures on the formation of polyplexes was evaluated. Additionally, we examined different total flow rates for each chip again, but both pumps were operated with the same individual flow rate. Polyplexes were about 225 nm in size, independent of the chip used for preparation (Fig. 3A). Polyplexes prepared by pipetting were slightly larger than those prepared by microfluidics and the standard deviation of the particle size was higher for pipetted polyplexes than for those prepared with a longer delay chip.

Polydispersity indices went under a certain variation for different flow rates, especially when chips with a short delay channel length after the Y-part were used (Fig. 3B). Using chips with longer delay channels the PdI was more uniform, independent from the flow rate. As explained above, mixing in microfluidic systems depends on diffusion. Lower flow rates allow longer times for the polyplexes to form, leading to a decreased PdI. The greater the length of the mixing channels, the lesser the influence of the flow rate, because diffusion was enhanced due to the longer mixing area. Furthermore, mixing times were also increased due to the longer delay channel with several loops, which the fluids had to pass.

These results consolidated our initial assumption from the first screening experiments (Fig. 4), that the flow rates had more influence on the PdI than on the particle size. Others also determined time to be a significant factor in polyplex formation. Tőke *et al.* found polyplexes at low N : P ratios required more time for formation than those at higher N : P ratios.[24]

Polyplex concentration in solution was reported previously to be a major obstacle for their stability (*e.g.* avoiding aggregation).[5] For different applications, *e.g. in vitro* or *in vivo* experiments, clinical trials or physicochemical characterization, a different polyplex concentration might be suitable. In the past polyplex aggregation has been observed when they were prepared at high concentrations.[5] Kapser *et al.* evaluated a scaled-up method for the preparation of linear PEI/DNA polyplexes and reported that their system was able to generate stable polyplexes at high concentrations.[11] It was thought that this might be possible with the lab-on-a-chip system described here as well and so the preparation of polyplexes in different concentrations was evaluated in comparison to the standard pipetting method.

Indeed, it was possible to prepare polyplexes in high concentrations using the lab-on-a-chip method (Fig. 5). Interestingly, with increasing concentration, the polyplex sizes

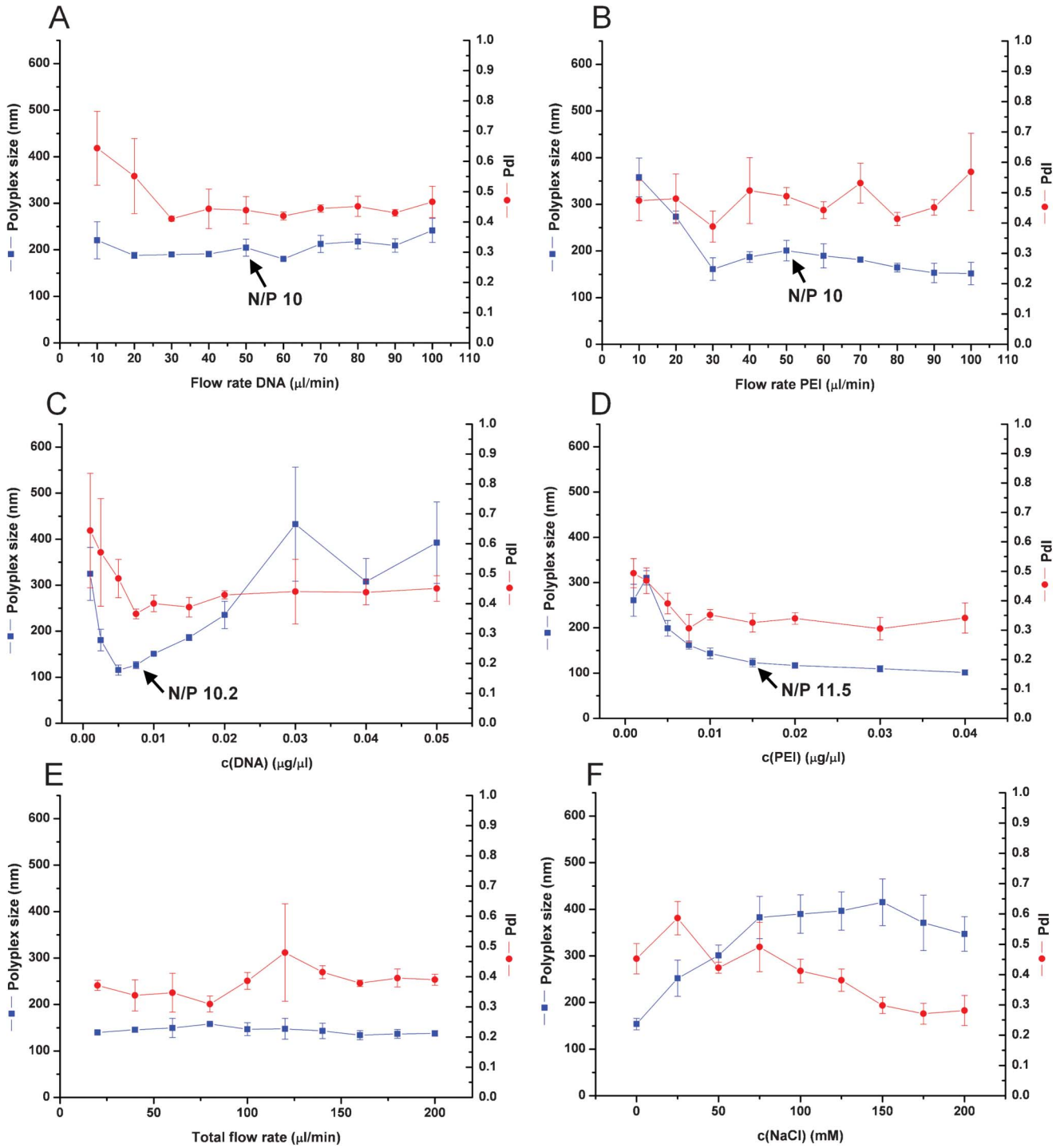

Fig. 4 Evaluation of different parameters influencing the formation of polyplexes prepared by microfluidic mixing on a chip. For all experiments a chip with 50 mm delay after the Y-part was used (50–100 R3). (A) Variation of DNA flow rate, PEI flow rate was kept constant at 50 µl min^{-1}. (B) Variation of PEI flow rate, DNA flow rate was kept constant at 50 µl min^{-1}. (C) Variation of DNA concentration with constant PEI concentration and constant flow rate of 50 µl min^{-1} for both solutions. (D) Variation of PEI concentration with constant DNA concentration and constant flow rate of 50 µl min^{-1} for both solutions. (E) Variation of total flow rate. The same flow rate was applied for both solutions (N : P = 10). (F) Variation of NaCl concentration. A constant flow rate of 50 µl min^{-1} was applied for both solutions (N : P = 10).

were constant when prepared by microfluidics and increased when prepared by pipetting. This could be a major advantage of the microfluidic method, because polyplexes in different concentrations (for different applications) had almost the same size, while the size of pipetted polyplexes increased considerably at concentrations >100 µg ml^{-1}.

The PdI decreased with increasing concentration for both preparation methods and the results were therefore in contrast to

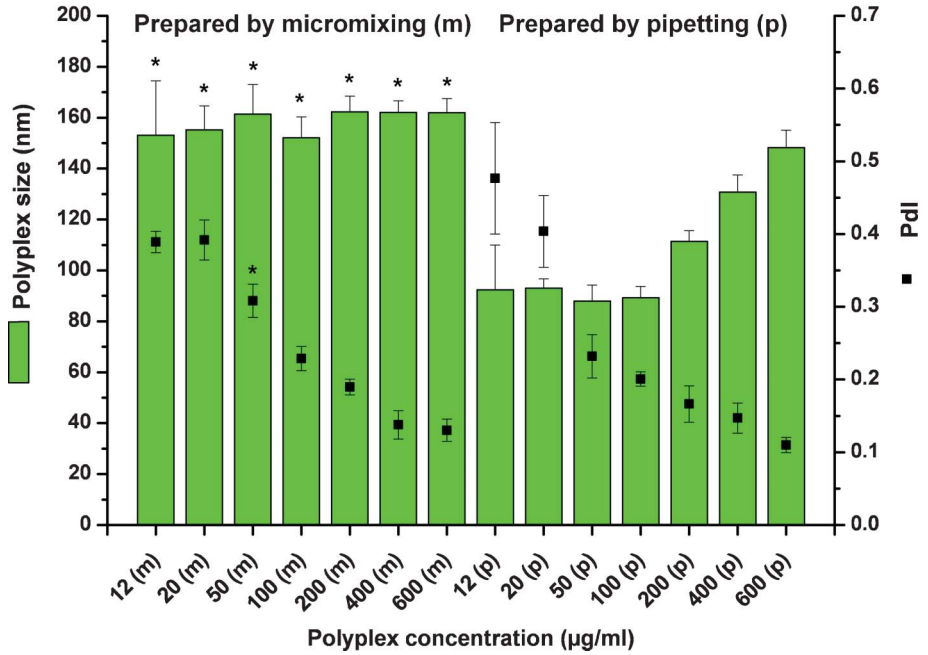

Fig. 5 Effect of polyplex concentration on the size and polydispersity index of PEI–DNA polyplexes prepared by microfluidics (m) or by pipetting (p). For microfluidic preparation with a chip 100–100 R3 a constant flow rate of 50 µl min^{-1} was applied for both solutions (N : P = 10). * $p < 0.05$.

those obtained of Kasper *et al.*, who observed increasing PdI with increasing concentrations.[11] In contrast, we did not observe an increase in particle size for chip-prepared polyplexes, and they reported increasing particle sizes with increasing concentrations for preparation with pumps and a T-connector. An explanation for these differences in particle sizes could be the difference of the preparation methods. The use of a chip with delay loops showed preferable mixing properties and seemed to be very suitable for the production of well defined polyplexes at different concentrations. The chip design with delay loops provided a longer mixing time than mixing by a T-connection only. The differences in PdI could be due to the use of a different polymer. While linear PEI(22 kDa) used by Kasper *et al.*[11] was a relatively well defined polymer, the branched PEI(25 kDa) used in the present study was more polydisperse and therefore thought to form less defined polyplexes. Polycation chain length was shown to play a crucial role in gene transfection,[25] what could be another reason for the differences observed.

Because the heterogeneity of commercially available branched PEI is a general problem, Bieber *et al.* developed a fractionation procedure for PEI(25 kDa) demonstrating that a low molecular weight fraction enhanced gene transfection efficiency.[17] Later this fraction was shown to be a suitable vector for small interfering RNA (siRNA) delivery as well.[26] The hypothesis was that a more defined fraction of PEI (25 kDa) should also form more homogenous polyplexes when prepared by microfluidics. PEI (25 kDa) was fractionated accordingly and the molecular weight of these fractions was estimated to be between 4000 and 10 000 Da.[26] Fig. 6 shows the elution profile of PEI (25 kDa) in the fractionation experiments. The marked fractions of lower molecular weight were pooled and the PEI content was calculated based on the copper assay results. The pooled fractions were designated as PEI(25 kDa) low molecular weight fractions (PEI(25 kDa) LMW fractions).

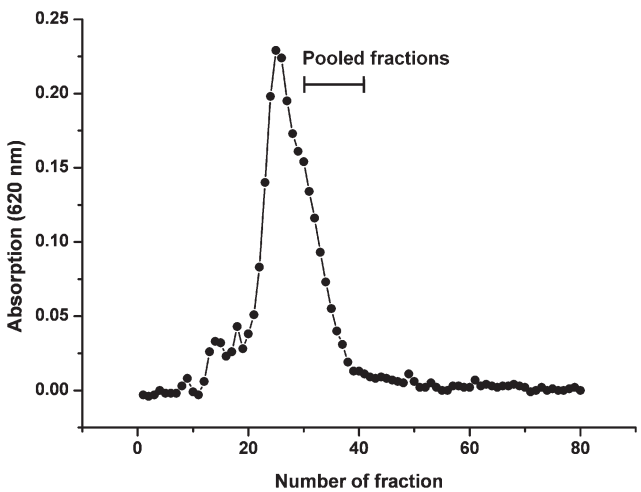

Fig. 6 Fractionation of PEI(25 kDa) using gel filtration. Fractions of 1 ml were analyzed by copper assay and absorption at 620 nm was measured. Low molecular fractions were pooled as indicated.

Polyplexes with pDNA and this polymer fraction were prepared. Additionally, a commercially available low molecular weight PEI (5 kDa) and a promising new targeted vector, transferrin-modified PEI (5 kDa) (Tf-PEI (5 kDa)),[16] were compared to evaluate the suitability of the method for the preparation of different polyplexes. The microfluidic method was compared with the standard pipetting method.

Experimental results (Fig. 7) showed the particles obtained by micromixing method not to be smaller than those prepared by pipetting (except Tf-PEI (5 kDa)). The polydispersity indices of micromixed polyplexes were lower than those of the pipetted polyplexes (significantly lower for PEI (25 kDa) and Tf-PEI (5 kDa)), which was our assumption. The size of polyplexes

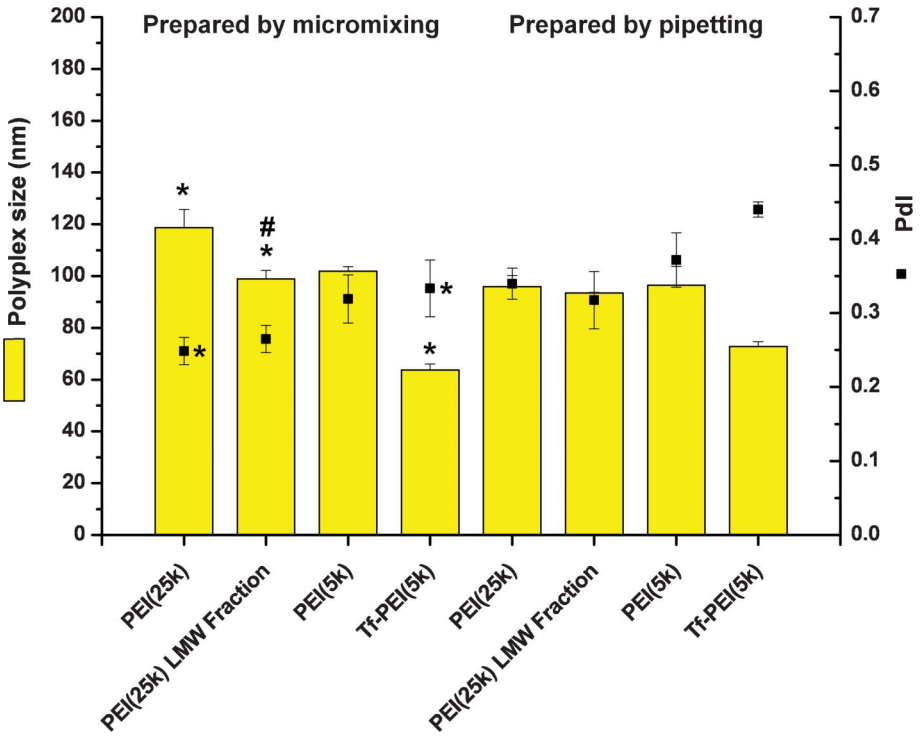

Fig. 7 Preparation of pDNA polyplexes with different polymers by microfluidics and by the standard pipetting method. Microfluidic preparation was done with the 100–100 R3 chip; a flow rate of 50 µl min^{-1} for both solutions and a polyplex concentration of 50 µg ml^{-1} were applied (N : P = 10). * $p < 0.05$ in comparison to preparation by pipetting. # $p < 0.05$ in comparison to PEI (25 kDa).

formed with PEI (25 kDa) LMW fractions by micromixing was significantly lower than the size of those with PEI (25 kDa).

Because one of the objectives of this study was the development of an all-purpose polyplex preparation method, we evaluated the use of different polymers as shown above. Furthermore, we were interested in the suitability of our method to assemble polyplexes with other nucleic acids than pDNA. Hence, we prepared polyplexes using the lab-on-a-chip method with siRNA and mRNA as well. The results (Fig. 8) showed, that the method was also suitable for the preparation of siRNA and mRNA polyplexes. To our knowledge, this study is the first one describing a microfluidic lab-on-a-chip method for the preparation of siRNA and mRNA polyplexes. As expected, differences in particle size and PdI could be observed between the polyplexes with different nucleic acids. While sizes are less different, PdIs are pretty much higher for siRNA polyplexes. The order of particle sizes and PdI for different complexing polymers was comparable for DNA, siRNA and mRNA. The differences

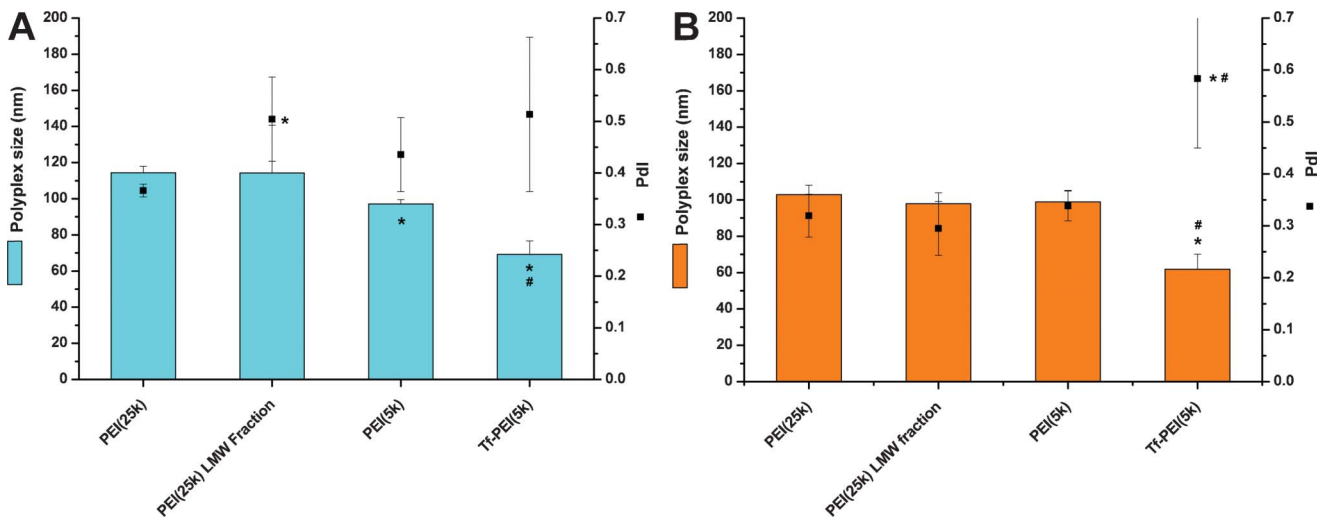

Fig. 8 Preparation of (A) siRNA and (B) mRNA polyplexes with different polymers by the microfluidic method. The preparation was done with the 100–100 R3 chip; a flow rate of 50 µl min^{-1} for both solutions and a polyplex concentration of 50 µg ml^{-1} were applied (N : P = 10). * $p < 0.05$ in comparison to PEI (25 kDa), # $p < 0.05$ in comparison to PEI (5 kDa).

could be caused by the different sizes of these nucleic acids. Smaller nucleic acids are discussed to form larger polyelectrolyte complexes.[27] Because their compaction due to electrostatic interaction might be less good than that of larger nucleic acids like pDNA, the uniformity of the polyplexes could be reduced and therefore the PdI might increase. This was confirmed by our results, where the PdI of siRNA complexes was generally higher than that of DNA and mRNA complexes for most of the polymers examined.

Conclusion

In conclusion, we were able to develop a novel lab-on-a-chip microfluidic method for the preparation of polyplexes with different (targeted) polymers and nucleic acids. This preparation method was shown to provide the possibility for preparation of various polyplexes and has a favourable modular construction,[28] what allows individual modifications for the intended purpose.

Polyplexes prepared by this method were shown to be more uniform than those prepared by standard pipetting method and the method was applicable for a broad range of concentrations. Well-defined polyplexes would be an important condition for future *in vivo* and clinical studies with such gene delivery systems and, therefore, an automated method like the described one will be a promising preparation alternative. Although this lab-on-a-chip method is a miniaturization of the standard method, it might be suitable for the manufacturing of larger volumes as well, because it is a continuous method and could be further developed or up-scaled as well.

Acknowledgements

CureVac GmbH (Tübingen, Germany) is gratefully acknowledged for providing messenger RNA (RNActive®).

References

1 O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix and J. P. Behr, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 7297–7301.
2 S. Nimesh, N. Gupta and R. Chandra, *J. Biomed. Nanotechnol.*, 2011, **7**, 504–520.
3 A. Kichler, *J. Gene Med.*, 2004, **6**, S3–10.
4 M. Beck-Broichsitter, C. Ruppert, T. Schmehl, A. Guenther, T. Betz, U. Bakowsky, W. Seeger, T. Kissel and T. Gessler, *Nanomed.: Nanotechnol., Biol. Med.*, 2011, **7**, 341–350.
5 J. Y. Cherng, H. Talsma, R. Verrijk, D. J. Crommelin and W. E. Hennink, *Eur. J. Pharm. Biopharm.*, 1999, **47**, 215–224.
6 C.-Y. Lee, C.-L. Chang, Y.-N. Wang and L.-M. Fu, *Int. J. Mol. Sci.*, 2011, **12**, 3263–3287.
7 P. Tabeling, *Lab Chip*, 2009, **9**, 2428–2436.
8 V. Hessel, H. Löwe and F. Schönfeld, *Chem. Eng. Sci.*, 2005, **60**, 2479–2501.
9 E. A. Mansur, M. Ye, Y. Wang and Y. Dai, *Chin. J. Chem. Eng.*, 2008, **16**, 503–516.
10 L. Y. Yeo, H. C. Chang, P. P. Chan and J. R. Friend, *Small*, 2010, **7**, 12–48.
11 J. C. Kasper, D. Schaffert, M. Ogris, E. Wagner and W. Friess, *Eur. J. Pharm. Biopharm.*, 2010, **77**, 182–185.
12 C. G. Koh, X. Kang, Y. Xie, Z. Fei, J. Guan, B. Yu, X. Zhang and L. J. Lee, *Mol. Pharmaceutics*, 2009, **6**, 1333–1342.
13 C. G. Koh, X. Zhang, S. Liu, S. Golan, B. Yu, X. Yang, J. Guan, Y. Jin, Y. Talmon, N. Muthusamy, K. K. Chan, J. C. Byrd, R. J. Lee, G. Marcucci and L. J. Lee, *J. Controlled Release*, 2010, **141**, 62–69.
14 J. Wang, Y. Zhan, V. M. Ugaz and C. Lu, *Lab Chip*, 2010, **10**, 2057–2061.
15 O. Zelphati, C. Nguyen, M. Ferrari, J. Felgner, Y. Tsai and P. L. Felgner, *Gene Ther.*, 1998, **5**, 1272–1282.
16 H. Debus, A. Kilic, M. Schiller, H. Garn, H. Renz, O. M. Merkel and T. Kissel, 2012, **in preparation**.
17 T. Bieber and H. P. Elsasser, *Biotechniques*, 2001, **30**(74-77), 80–71.
18 A. von Harpe, H. Petersen, Y. Li and T. Kissel, *J. Controlled Release*, 2000, **69**, 309–322.
19 W. F. Fang, M. H. Hsu, Y. T. Chen and J. T. Yang, *Biomicrofluidics*, 2011, **5**, 14111.
20 Y. P. Ho, H. H. Chen, K. W. Leong and T. H. Wang, *Nanotechnology*, 2009, **20**, 95103.
21 L. Leclercq, M. Boustta and M. Vert, *J. Drug Targeting*, 2003, **11**, 129–138.
22 T. Etrych, L. Leclercq, M. Boustta and M. Vert, *Eur. J. Pharm. Sci.*, 2005, **25**, 281–288.
23 J. Nguyen, X. Xie, M. Neu, R. Dumitrascu, R. Reul, J. Sitterberg, U. Bakowsky, R. Schermuly, L. Fink, T. Schmehl, T. Gessler, W. Seeger and T. Kissel, *J. Gene Med.*, 2008, **10**, 1236–1246.
24 E. R. Tőke, O. Lorincz, E. Somogyi and J. Lisziewicz, *Int. J. Pharm.*, 2010, **392**, 261–267.
25 Y. Yue, F. Jin, R. Deng, J. Cai, Z. Dai, M. C. Lin, H. F. Kung, M. A. Mattebjerg, T. L. Andresen and C. Wu, *J. Controlled Release*, 2011, **152**, 143–151.
26 S. Werth, B. Urban-Klein, L. Dai, S. Hobel, M. Grzelinski, U. Bakowsky, F. Czubayko and A. Aigner, *J. Controlled Release*, 2006, **112**, 257–270.
27 D. J. Gary, N. Puri and Y.-Y. Won, *J. Controlled Release*, 2007, **121**, 64–73.
28 S. Haeberle and R. Zengerle, *Lab Chip*, 2007, **7**, 1094–1110.

