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Parallel High-Throughput Screening of Polymer Vectors for Nonviral Gene Delivery: Evaluation of Structure–Property Relationships of Transfection

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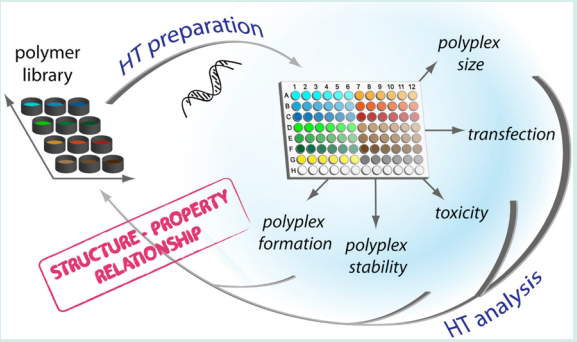
Supporting Information

ABSTRACT: In recent years, "high-throughput" (HT) has turned into a keyword in polymer research. In this study, we present a novel HT workflow for the investigation of cationic polymers for gene delivery applications. For this purpose, various poly(ethylene imine)s (PEI) were used as representative vectors and investigated via HT-assays in a 96-well plate format, starting from polyplex preparation up to the examination of the transfection process. In detail, automated polyplex preparation, complex size determination, DNA binding affinity, polyplex stability, cytotoxicity, and transfection efficiency were performed in the well plate format. With standard techniques, investigation of the biological properties of polymers is quite time-consuming, so only a limited number of materials and conditions (such as pH, buffer composition, and concentration) can be examined. The approach described here allows many different polymers and parameters to be tested for transfection properties and cytotoxicity, giving faster insights into structure–activity relationships for biological activity.



KEYWORDS: *high-throughput screening, transfection, nonviral gene delivery, polyplex stability, poly(ethylene imine), heparin, combinatorial workflow*

INTRODUCTION

Nonviral gene delivery (transfection) methods are of great interest for research and clinical applications. The use of cationic polymers as nonviral vectors to form complexes (polyplexes) with negatively charged plasmid DNA (pDNA) has long been explored as a safer and more controllable alternative to the use of possible infectious viral vectors.[1,2] For the evaluation of polymers as transfection agents, two main aspects must be considered: the efficiency of gene delivery with subsequent reporter gene expression and cytotoxicity.[3] Biophysical properties, such as polyplex size, surface charge, and binding affinity between the polymer and the genetic material play crucial roles in the required cellular uptake.[4,5] The binding within the interelectrolyte complex of polymer and pDNA has to be strong enough to protect the pDNA but must be reversible to release the pDNA inside the cells.[6,7] While much progress has been made, there is still an insufficient knowledge of how polymers should be constructed to be highly efficient and safe gene delivery vectors.[8,9]

This lack of predictability results in part from the great diversity of polymer classes and methods reported in the literature, which are difficult to compare to each other. For instance, transfection protocols differ notably for different cells and media, and different polymer solutions and buffers are used in the preparation of polyplexes.[10,11] While some examples have been used for in vitro applications and biotechnology research for decades, no polymer-based transfection agent has been approved for clinical use.[12−14]

The development of robotic techniques for the preparation of polymeric materials provides an opportunity for the high-throughput (HT) synthesis and characterization of cationic polymers in this context.[15−20] Using this synthetic approach, polymer properties such as molar mass, functional groups, architecture, and the combination of different monomers in

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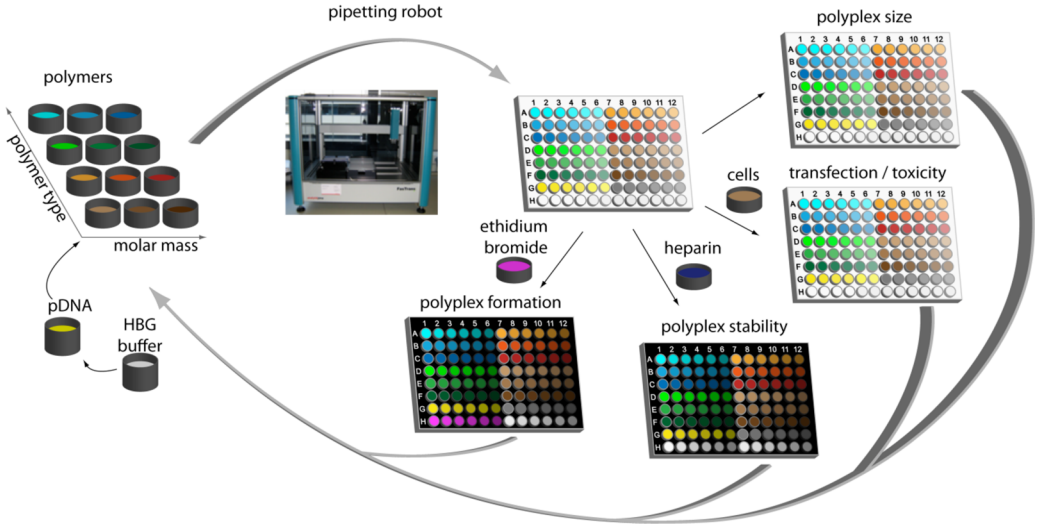
Scheme 1. Workflow of the High-Throughput Transfection Studies for Structure−Property Evaluations Concerning Molar Ratio, Size, Polyplex Formation, Polyplex Stability, Release, Transfection Efficiency, and Cytotoxicity



statistic or block copolymers can be altered, yielding systematic polymer libraries, which enable the elucidation of structure−property relationships.[15−23] Unfortunately, rapid methods for biological evaluation have not been hyphenated with efficient automated synthesis to construct a combinatorial HT workflow.[24−27] For example, binding affinity and polyplex stability have commonly been assessed by agarose gel electrophoresis, which is not well suited to HT screening. The use of an intercalating dye to establish binding affinities can provide an alternative compatible with a microtiter plate format.[28] Transfection and cytotoxicity assays can be similarly performed in multiwell plates with repeating samples to reduce measurement mistakes. Pioneers in this type of HT screening of a wide range of polymers as transfection agents have been Langer and co-workers (synthesis and transfection efficiency)[29] and Massing and co-workers (lipofection transfection efficiency and toxicity).[30]

We describe here a simple and powerful combinatorial high-throughput workflow that combines polyplex formation and biological screening (Scheme 1). It starts with the automated polyplex preparation via pipetting robots and continues with a parallel and HT analysis of analytical and biological properties of size, binding affinity, stability, transfection efficiency, and toxicity. We show that the novel workflow is applicable to a variety of polymer systems and conditions, allowing for fast and efficient screening of important vector parameters, such as polyplex formation, pDNA release, cytotoxicity, and transfection.

RESULTS AND DISCUSSION

Poly(ethylene imine) (PEI), the most prominent cationic polymer and most efficient transfection agent for pDNA in vitro, was used to validate the method.[31,14] Linear PEI (LPEI), obtained from poly(2-ethyl-2-oxazoline)s of different molar masses, was prepared.[32,33] By application of automated microwave synthesizers, poly(2-ethyl-2-oxazoline)s can be obtained within 10 min and converted into PEI within 1 h by acidic hydrolysis.[32] These cationic PEI polymers offer the advantage to be molecularly designed in a highly reproducible manner for specific applications in pharmacy or biotechnology. Commercially available branched PEI (BPEI) materials were also used in this study.

Evaluation of an Appropriate Buffer System. The formation of polyplexes from cationic polymers and anionic genetic material is driven by electrostatic interactions and a gain of entropy.[34] Thus, ionic strength, pH, and the final polyplex concentration have a major impact on the complexation behavior and the resulting polyplex size.[35,36] For the ex cellular characterization, polyplexes are often prepared in high ionic strength buffers, such as 150 mM sodium chloride (NaCl) or buffer systems using phosphate (PBS) or TRIS (TBS). Such high ionic strength media can have a negative impact on particle size and stability and lead to fast polyplex aggregation.[36,37] Thus, a low ionic strength 20 mM HEPES buffer with 5% glucose for physiological osmolarity (HBG buffer) was examined for polyplex preparation in a HT manner, as has been previously done for transfection.[37] Preliminary studies with linear PEI_{600} revealed that smaller polyplexes were formed in HBG.[35] A lower tendency to aggregate over time compared to physiological salt solutions (150 mM NaCl) was observed if the polyplexes were prepared in HBG.[38−40] Our measurements showed $LPEI_{600}$ polyplexes to exhibit no aggregation over 2 h in HBG (see Supporting Information), no aggregation or particle growth before and after the addition to serum containing culture media.[36−38] Furthermore, HBG buffer can be used for zeta potential measurements[41] in this concentration range as well as for electron microscopic evaluations, where salts cause electrophoresis or artifacts, respectively. Consequently, HBG was selected as most appropriate buffer system for HT studies and was used here for all polyplex preparations and analytical investigations.

Polyplex Preparation Using Pipetting Robots. A standard liquid handling robot was used for automated preparation of polyplexes from cationic polymers and DNA, similar to reports of robotic production of polymeric nanoparticles.[42] The benefit of such pipetting systems is the ability to systematically alter different parameters, such as polymer concentration, pH, or buffer composition.[43] Automated deposition of a buffered pDNA solution into wells containing various buffered cationic polymer solutions at desired concentrations was performed. While the reverse

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addition (pipetting polymer to DNA solution, vortexing after polymer addition) is the more conventional method,[1] giving better transfection results,[44] we observed similar outcomes in preliminary experiments using $LPEI_{600}$ and $BPEI_{600}$ (see Supporting Information) and in scattered tests of the HT method (data not shown). This may be due to more reversible interelectrolyte formation in the low ionic strength HBG buffer compared to high ionic strength buffers used previously.

To evaluate the dependence of polyplex properties on the nature of the polymers and preparation conditions, various cationic LPEI and BEI with varied degree of polymerization (DP = 20, 200, and 600) were used to form polyplexes with pDNA. Besides the molar mass and architecture, several nitrogen (polymer) to phosphate (DNA) ratios (N/P = 2.5, 5, 10, and 20) were applied. To this end, pDNA solution was added to a dilution series of polymer solutions, and the resulting suspensions were directly mixed by repetitive suction and release. After polyplex formation, the prepared suspensions were distributed automatically into different well plates for parallel analysis studies.

Investigation of Polyplex Size and Stability. The polyplex size allows a first hint of the polymer's capability to be used as a transfection agent, since polyplexes larger than 500 nm are known to show relatively poor uptake.[45] For this purpose, the polyplexes were first analyzed on a dynamic light scattering (DLS) plate reader.[46] As shown in Figure 1, all

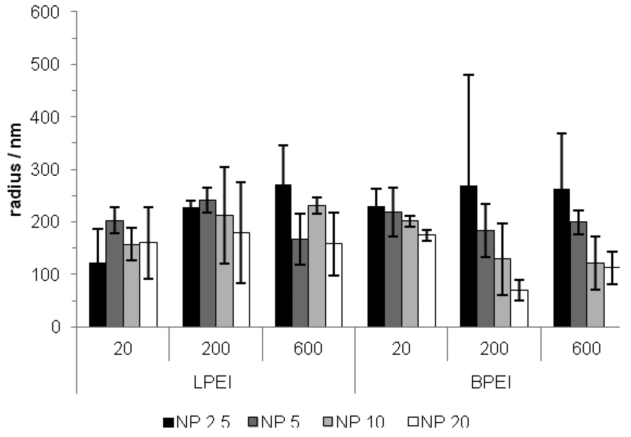

Figure 1. Hydrodynamic radii of polyplexes prepared using the pipetting robot. The values represent the mean ($n \geq 3$) of each polymer at different N/P ratios. PDI^P values of 0.09 to 0.5 were found.

polyplexes exhibited an average radius of less than 270 nm, with materials mixed at N/P ratios above 5 showing smaller radii. The smallest size (70 nm radius) was obtained for $BPEI_{200}$. It should be noted that the HT-DLS analysis data tended to report larger radii than measurements performed with a single-beam DLS instrument (see Supporting Information). HT-DLS results should be always considered with care, and we consider them informative only in a relative sense, to establish the potential of polymers to form nanoscaled polyplexes and gain information about their stability in comparison to standard polymer controls. Our data revealed three tendencies, also described in the literature: (i) increasing N/P ratios gave rise to smaller polyplexes, (ii) BPEI with higher DPs showed a stronger size dependency compared to LPEI, and (iii) BPEI condensed DNA into smaller particles compared to LPEI.[31] Interestingly, no systematic influence of the degree of polymerization or the molar mass on polyplex size was observed under the chosen conditions.

Fluorescence Displacement Assay. Considering the interpretation of transfection results the determination of the binding affinity of the polymers to the genetic material is of vital importance. As previously mentioned, the binding of a polyplex is at its optimal state when having a strong and reversible interaction. The required N/P ratio to form polyplexes were either done by usage of gel retardation assays or by fluorescence measurement of intercalating dyes, such as ethidium bromide (EB) or Pico Green. For an HT screening application, the gel retardation method is not suitable in a 96-well plate format, thus, the fluorescence displacement assay with EB (EBA) was chosen. Commonly, the binding of EB with pure pDNA leads to a high fluorescence signal. However, provided that the pDNA forms interelectrolyte complexes with the polymers, the displacement of dyes leads to a decrease of fluorescence signals. In Figure 2, the fluorescence signals

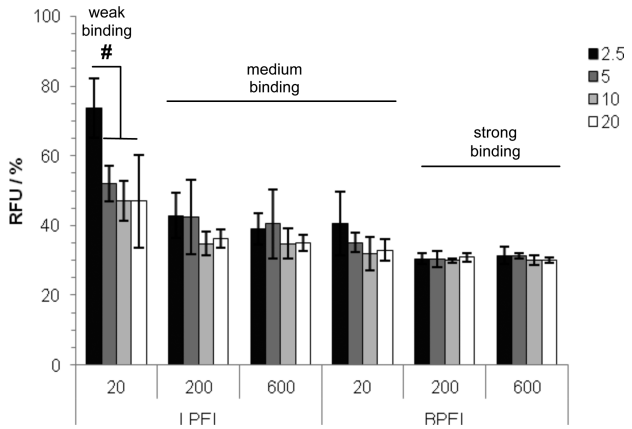

Figure 2. Fluorescence displacement assay using LPEI and BPEI with varying DP. The RFU of pure pDNA represent 100% RFU. N/P ratios of 2.5 up to 20 were studied using EB as intercalating agent. The values represent the mean ± S.D., $n \geq 3$, # indicate significant statistical difference (ANOVA, $p < 0.05$).

(RFU) of all PEI polymers with increasing N/P ratio are illustrated. It was found that $BPEI_{200}$ and $BPEI_{600}$ reached a comparable RFU of around 30.5 ± 1.4% ($p > 0.5$) indicating a strong DNA binding. Furthermore, the higher molar mass LPEIs ($LPEI_{200}$ and $LPEI_{600}$) along with $BPEI_{20}$ revealed comparable RFUs in the range of 37.1 ± 6.2% ($p > 0.1$). The weakest binding was obtained with $LPEI_{20}$ also showing the strong dependence of N/P. In particular, polyplexes formed at N/P 20 revealed a mediate RFU of 48.7 ± 8% ($p > 0.5$) in comparison to 73.8 ± 8.5% at N/P 5. The findings accredit that the binding affinity depends on the molar mass and the architecture of the polymer as well as on the N/P ratio applied. The relationship between the binding affinity and the molar mass of the polymer increases in a proportional manner. In addition, a higher binding affinity of branched structures (BPEI) was detected in comparison to linear architectures.[8,31] The literature[47,48] reports similar trends and confirms the possible analysis of polyplexes by this HT assay. Moreover, identical tendencies were obtained for this particular handmade assay using polyplexes of linear PEIs (see Supporting Information). At this point of the workflow, one should note that after performing size measurements and binding affinity assays, it is possible to exclude nonsuitable polymers as

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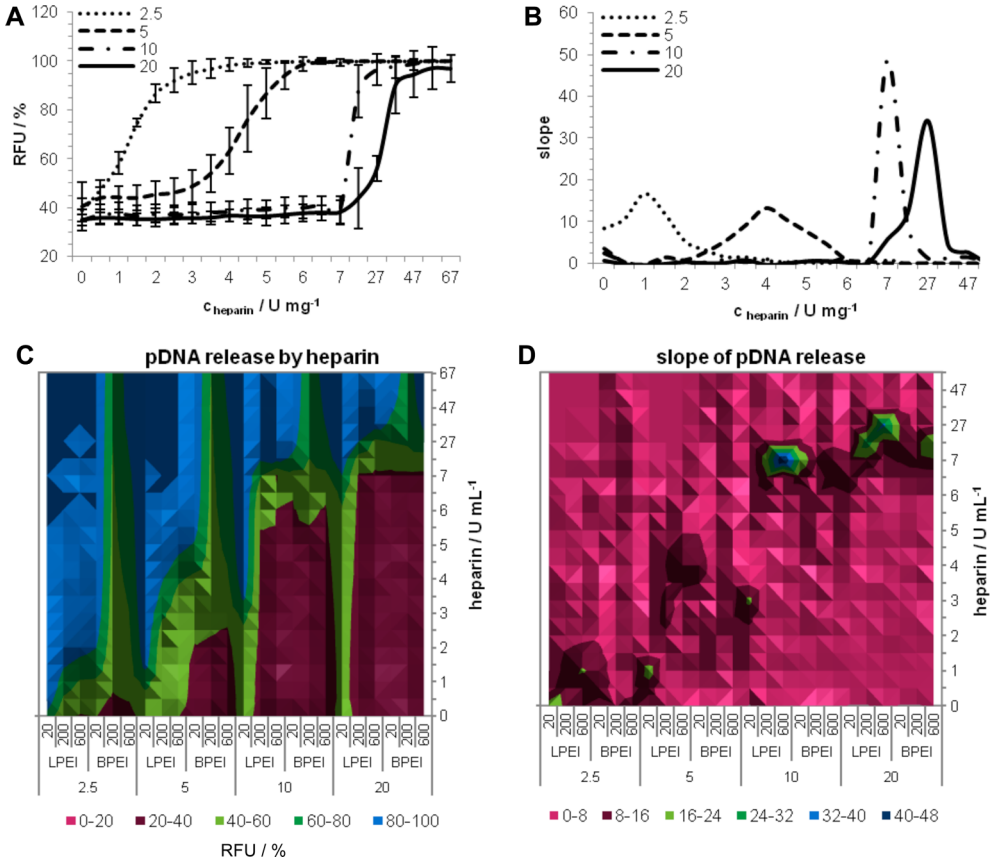

Figure 3. pDNA release of polyplexes after titration with heparin. Release of pDNA was measured by incubation of polyplexes with increasing heparin concentrations. (A) RFU of polyplexes prepared from $LPEI_{600}$ at different N/P ratios and increasing heparin concentrations. (B) Slope of RFU of $LPEI_{600}$ polyplexes at different N/P ratios. (C) RFU of all polyplexes at different N/P ratios and increasing heparin concentrations. Color represents the RFU. (D) Slope of RFU of all polyplexes at different N/P ratios. Color represents the slope. The values represent the mean ± S.D., $n \geq 3$.

transfection agents, which showed undesired interaction such as aggregation or no polyplex formation.

DNA Release. Subsequent to the determination of binding affinity, the release of pDNA from the polyplexes was investigated using the heparin assay. Heparin is a polyanion and it was reported to be a good competitor to negatively charged pDNA.[48] As a result of the polymer-heparin interaction, the pDNA is released and EB is repeatedly intercalating into pDNA leading to increased fluorescence intensities. Studies using heparin are often quantified via gel retardation assays or applying only one N/P ratio, which would potentially lead to misinterpretations. In particular for in vitro cultivations of adherent cells, the polyplex concentration at the cell membrane at the beginning of the transfection and after incubation differs. The explanation for this behavior could be justified by the polyplex sedimentation process.[8,49,50] For a more trustworthy outcome, all polyplex suspensions were titrated automatically against two heparin stock solutions to determine the critical heparin concentration at different N/P ratios. Using this approach, a wide range of heparin concentrations (n = 20) could be tested for one sample. The results obtained from the performed assay are displayed in detail for $LPEI_{600}$ (Figure 3A) and for all polymers and N/P ratios in (Figure 3C). As expected, the release of pDNA detected by RFU was dependent on the heparin concentration. Moreover, it was explored that for the release of total pDNA at higher N/P ratios, an increased amount of heparin was required. This can be explained by the fact that the amount of noncomplexed free polymer was increased at high N/P ratios, whereas the amount of complexed polymer remained constant.[51] Thus, by the addition of heparin to polyplex suspensions at high N/P ratios, first the free polymers complex with the heparin and no pDNA was released. Unless the critical concentration of heparin was met, the pDNA was not released. For an improved comparability, the inflection point of the titration curves in Figure 3A and C was defined as the critical heparin concentration (HC_{50}) and implemented as a representative value of the concentration at which 50% of the complexed pDNA was released (Figure 3B and D). The correlation between the N/P ratio and the heparin concentration was an apparent observation and confirmed already published trends.[48] However, our findings underline the relation between the architecture of PEI and the ability to release pDNA.

Polyplexes prepared from BPEIs showed higher HC_{50} values in comparison to the LPEIs (indicated by larger purple areas in particular at N/P 2.5 and 5, Figure 3C and D). Furthermore, the polyplexes prepared with the $LPEI_{20}$ exhibited an early release of the pDNA at low heparin concentration in contrast to its branched analog ($BPEI_{20}$) and the linear PEIs with higher molar masses ($LPEI_{200}$ and $LPEI_{600}$). A flagrant correlation could be made of these with the weak binding affinity (Figure 2).

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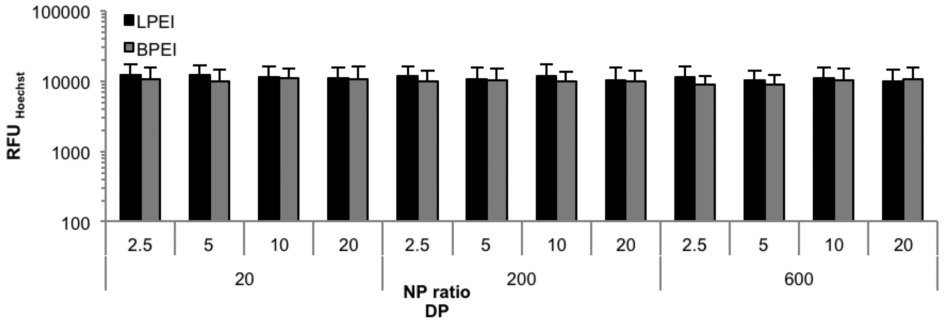

Figure 4. Investigation of cytotoxicity. The viability of cells after incubation of the polyplexes up to N/P 20. Nontreated cells served as controls and gave comparable results. The bottom of 96-well plates were measured at Em_{350}/Ex_{461} (Hoechst 33324).

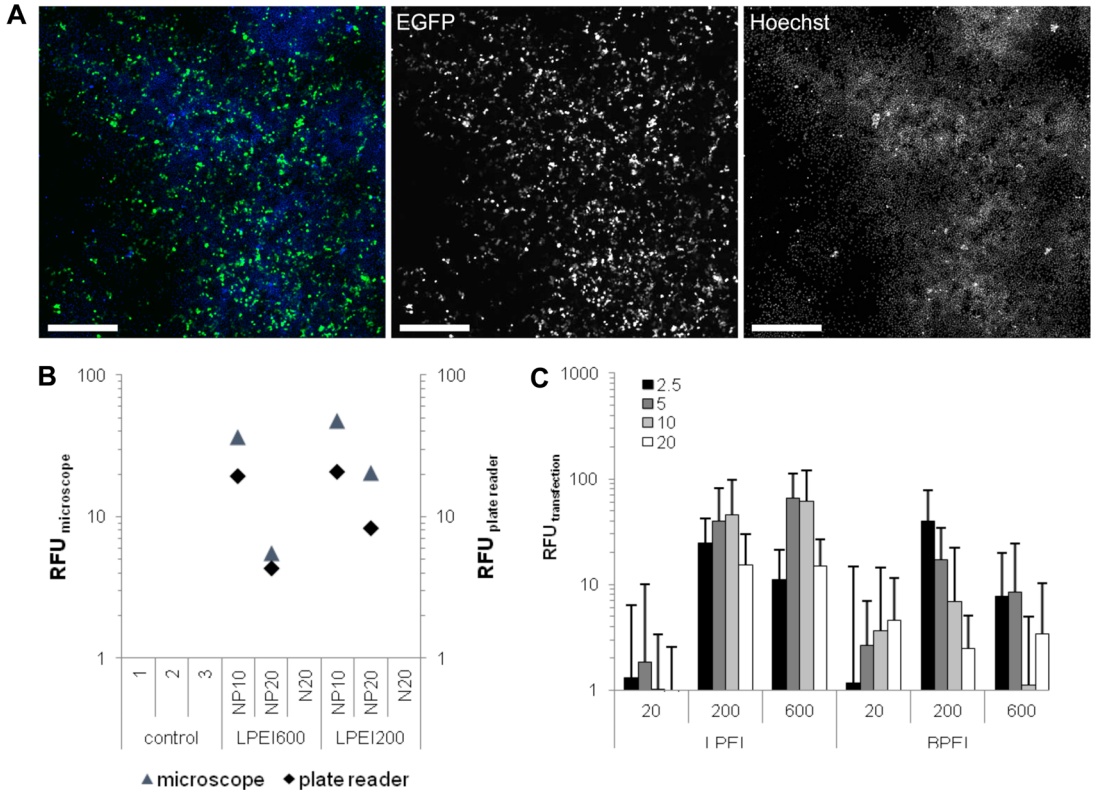

Figure 5. Transfection efficiency by microscopic evaluation and fluorescence intensity measurements. (A) HEK cells transfected with EGFP coding pDNA and $LPEI_{600}$ at N/P 10. Cell nuclei were stained with Hoechst 33324 (blue). Scale bare indicates 500 μm. (B) Correlation of the microscopic evaluation of EGFP content determined ($RFU_{microscope}$) and bottom measurements using a plate reader ($RFU_{plate\ reader}$). Three control wells, where cells were not transfected, as well as cells only incubated with the polymer at concentrations correspond N/P 20 (N20) showed no RFU. (C) Transfection efficiency and number of cells transfected with a pipetting robot in a 96-well plate. Values represent the mean ± S.D., $n \geq 3$.

Cytotoxicity. To study the cytotoxicity of the polyplexes, HEK cells used as for transfection experiments, were seeded in 96-well plates and incubated for 24 h with the prepared polyplex suspensions. Afterward, the viability of the cells was detected by staining with Hoechst 33324. This dye crosses of the cell membrane and stains the chromosomal DNA of attached cells. Subsequently, the fluorescence was measured utilizing the fluorescence plate reader. The obtained RFU signals of Hoechst of all treated cells are presented in Figure 4. No indication for cytotoxic effects of the polyplexes was found considering the fact that the obtained values were comparable to nontreated cells (ANOVA, $p > 0.05$).

As the polyplexes exhibited a lower cytotoxicity than the single polymers, due to neutralized cationic groups, the toxicity of these polyplexes at N/P 20 would be a criterion for knock out. However, for a comprehensive analysis, the polymers were also screened with concentrations up to 72 μg mL^{-1}, correlating to N/P 500 (DP 20 and 600, see Supporting Information). A relationship was elucidated between the increasing DP of the cationic polymers and the higher cytotoxicity level, which is in accordance to literature where no significant difference between linear and branched PEI was observed.[3,31] Interestingly, the polymers with the lowest DP showed no cytotoxicity at all investigated concentrations (see Supporting Information).

Transfection Efficiency. The transfection efficiency of the polyplexes was quantified using EGFP as reporter protein. For HT screening, the studies regarding the transfection efficiency were performed with a fluorescence plate reader by automatic scanning of the bottom area of the wells and complemented by

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microscope analysis. In Figure 5A, a representative overview of the cells (blue) transfected with $LPEI_{600}$ (green) is portrayed. Microscopic analysis and, in particular, subsequent data processing is not appropriate and efficient enough for a rapid HT screening, thus the quantification of EGFP using a fluorescence plate reader were compared to the mean fluorescence in each picture (Figure 5B). Thereby, a good correlation between the microscopic analysis and the fluorescence plate reader results was demonstrated, proving the capability to screen the EGFP amount in a fast and facile manner, in contrast to flow cytometry or microscopy. In general, it can be stated that there are some decent advantages of a fluorescence screening with a plate reader compared to luciferase or galactose based assays, namely, (i) an easy and cheap detection, (ii) the possibility to perform afterward single cell analysis by flow cytometry or microscopy of the same cells, and (iii) the fact that EGFP is a stable reporter protein.

The EGFP expression for all investigated PEI polymers are shown in Figure 5C. The polymers can be ranked from high to low transfection efficiency: $LPEI_{600}$ > $LPEI_{200}$ > $BPEI_{200}$ > $BPEI_{600}$ > $BPEI_{20}$ > $LPEI_{20}$, whereby the obvious increase in the standard deviation compared to flow cytometry measurements (see Supporting Information) must be taken into account. However, the HT investigation showed clear trends confirming a suitable approach to spot high potential candidates and to be subsequently investigated in depth. Thus, $LPEI_{20}$ revealed no transfection efficiency, while $LPEI_{600}$ shows the highest one, also confirmed by handmade polyplexes and the detection of EGFP by flow cytometry (see Supporting Information). This observation further verifies the potential of such a HT screening using a fluorescent plate reader for determination of the transfection efficiencies of polymers.

CONCLUSION

Since HT synthesis and characterization of polymers could be managed by synthetic robots and microwave synthesizers combined with subsequent automated characterization of the molecular properties, polymer libraries for biological applications can be prepared in a rapid manner.[15−20] So far, an efficient and fast HT screening of these polymers for gene delivery purposes regarding structure−property relationship was not possible. Herein, a solution for the biological screening for gene delivery applications has been presented. The discussed HT workflow enables a rapid analysis of polymer vectors in an automated way with respect to important polymer characteristics, such as molar mass, architecture, and N/P ratio. This supports the identification and evaluation of polymers with regard to their capability of efficient complexation, protection, and transfection efficiency. For instance, the described heparin assay can be used for 23 polymers at four different N/P ratios resulting in 92 samples plus controls (n = 1). Furthermore, the HT approach was applied and demonstrated the possible screening of the cytotoxicity and the transfection efficiency of the polyplexes. As expected, the study of the different PEI model polymers revealed that linear and branched PEI are noncytotoxic at the investigated concentrations, but with rising molar mass and polymer concentration the cytotoxic effect was increasing.[31] The polymeric architecture itself showed thereby no influence on the cell viability.[3] As per literature, at low molar masses the DNA binding affinity is influenced by the polymeric architecture, since $BPEI_{20}$ revealed a stronger pDNA binding than $LPEI_{20}$.[15,26] The obtained results indicated that PEIs with branched architectures and small molar masses have the highest potential to be used as gene vectors in vitro, as they offer the advantage of low cytotoxicity combined with high pDNA binding affinity. Beyond, the best transfection results were obtained for $LPEI_{600}$ and the $BPEI_{200}$.

In comparison with literature and handmade performances proof was established that the developed workflow is applicable for polymer systems. Furthermore, conditions enabling a fast and efficient screening in terms of important vector parameters, such as polyplex formation, transfection, and release were found. The possible screening of polymer libraries for the best transfection candidate will help to elucidate main polymer characteristics and to understand why some polymers are high performers and others not. Thus, an enhanced development of more efficient polymers and polyplexes can be realized.

EXPERIMENTAL PROCEDURES

Material. Ethidium bromide solution 1% was purchased from Carl Roth (Karlsruhe, Germany). AlamarBlue was obtained from Life Technologies (Darmstadt, Germany). If not otherwise stated, cell culture materials, cell culture media, and solutions were obtained from PAA (Pasching, Austria). Plasmid pEGFP-N1 (4.7 kb, Clontech, Mountain View, CA, U.S.A.) was isolated using Qiagen Giga plasmid Kit (Hilden, Germany). All other chemicals were purchased from Sigma Aldrich (Steinhausen, Germany) and are of analytical grade or better and used without further purification. Linear PEI was synthesized according to procedure described in literature.[32]

Polyplex Preparation Using Pipetting Robot. For an automated polyplex preparation, 100 μL buffered DNA solution (c = 15 μg mL^{-1}) were injected into wells that contain 300 μL of the desired polymer solution. As cationic polymers, linear PEI with a DP of 20, 200, and 600, as well as branched PEI with a DP of 20, 200, and 600 were applied. To achieve different polymer to DNA ratios (N/P ratios), a dilution series in HBG of four different polymer concentrations (N/P ratio 2.5, 5, 10, 20) was prepared using a pipetting robot from a polymer stock solution of c = 72 μg mL^{-1}. After addition of the DNA solution, the polyplex suspension was mixed five times by suction and release using 200 μL tips and incubated at least 20 min. Subsequently, 100 μL of each polyplex suspension were transferred into three different well plates for a detailed analysis studies. The following assays were performed up to 2 h after polyplex preparation.

ASSOCIATED CONTENT

Supporting Information

Additional experimental details, tables of SEC data and hydrodynamic radii of polyplexes, comparison of the conventional handmade ployplexes and HT polyplexes, figures showing transfection efficiency, fluorescence displacement and cytotoxicity, and hydrodynamic radii and PDI[P], and additional references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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