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Relating Chemical and Biological Diversity Space: A Tunable System for Efficient Gene Transfection

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Polyethyleneimine (PEI), a well-established nonviral transfection reagent, was combinatorially modified with varying proportions of methyl, benzyl, and n*-dodecyl groups to create a library of 435 derivatized polymers. Screening of this library for transfection, DNA binding, and toxicity allows systematic correlation of the biological properties of our polymers to their derivatizations. Combinations of derivatizations bring about a 100-fold variation in transfection efficiency between library members. The best PEI derivatives exhibit increases in transfection efficiency of more than 80-fold over unmodified PEI (up to 28 ± 7% of cells transfected) and rival commercial reagents such as Lipofectamine 2000 (21 ± 10%) and JetPEI (32 ± 5.0%). In addition, we can identify compounds that are specifically tuned for efficient transfection in CHO-K1 over Ishikawa cells and vice versa, demonstrating that the approach can lead to cell-type selectivity of at least one order of magnitude. This work demonstrates that multivalent derivatization of a polymeric framework can create functional diversity substantially greater than the structural diversity of the derivatization building blocks and suggests an approach to a better understanding of the molecular underpinnings of transfection as well as their exploitation.*

Introduction

A variety of gene delivery techniques are currently available, but no universally applicable and efficient solution has been found. Viral vectors remain the most efficient reagents to introduce DNA into living cells.[1,2] Nonviral vectors are potentially cheaper to produce and have fewer safety problems, but they are still orders of magnitude less efficient.[1,3–5] A variety of nonviral methods are in use, ranging from ballistics,[6] to magnetic[7] and other nanoparticles,[8] as well as synthetic transfection reagents.[4,5,9,10] The latter include cationic lipids[11] such as SAINTs,[12] the commercial Lipofectamine™ 2000, and polymers such as poly-L-lysines,[13] polyamidoamine[14] or polyamine dendrimers,[15] and polyethyleneimine (PEI).[16,17]

While the systematic variation of the chemical structure of a given molecule to improve its functional characteristics (e.g., in enzyme inhibition by small molecules) is a mainstay of research in medicinal chemistry, it has proven difficult to adapt transfection reagents efficiently for specific functions.[10,18] This is no doubt due to the complexity of the biological challenge, in which the DNA delivery agent must satisfy the requirements of a variety of transport processes.[19] These might include the lipoplex–cell surface interaction, internalization and membrane transfer, delivery into intracellular compartments, polymorphic transitions of lipoplexes (such as a membrane-destabilizing hexagonal phase),[11] endosomal release (avoiding lysosomal degradation), concomitant escape of DNA/RNA into the cytosol, and, finally, transport into the nucleus.[19–21] At this stage quantitative knowledge about these steps is limited; in particular, it is not clear which of these steps might be the bottleneck for any one class of delivery reagents. All these processes can potentially become rate-determining. Consequently the question of how structural variation of a transfection reagent affects each of these processes and influences the balance between them is naturally complex and a quantitative answer elusive.[10,20]

Only relatively recently have structure–activity relationships been discovered for small compound collections, such as gemini surfactants,[22] sunfish amphiphiles,[23] and cyclodextrin-containing polycations.[24] Higher-throughput library approaches have been used to screen reagent combinations whose efficacy cannot be predicted. For example, combinatorial solid-phase chemistry was used to synthesize a library of new cationic lipids and to improve their transfection properties.[25] Variation of the monomer building blocks of the cationic degradable polymers, poly-β-amino esters,[26,27] has given some insight into the biophysical properties necessary for transfection, such as particle size and surface charge.[28,29] This approach has yielded efficient transfection reagents that are already successfully used for gene transfer in cells.[30] There is potential for optimization at levels other than the backbone. For example, end-capping of poly-β-amino esters has improved transfection.[31] We are interested in further exploring the potential of these effects by studying relatively subtle changes to

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the surface of a polymer that can affect interactions with biological systems. To this end we have systematically explored the effect of PEI derivatization on the process of transfection.

The multivalency[32] of polymers such as PEI offers the potential for modification with many functional groups to create libraries of compounds with potentially diverse properties. PEI modification—by deacylation,[33] acetylation,[34] *n*-dodecylation,[35] or crosslinking[36]—has already been shown to improve efficacy moderately and to reduce toxicity.[5] By setting up a high-throughput system for in situ derivatization and screening, we can sample a focused area of chemical diversity ("chemical space") at high resolution to investigate the cumulative effect of minor variations. By superimposing the results of functional assays ("functional space") and chemical composition we hope to reveal more comprehensive relationships of derivatization and function in multidimensional chemical and biological descriptor space.[37]

A total of 435 different derivatives of branched PEI (25 kDa) were produced by modification with combinations of methylating, benzylating, and *n*-dodecylating reagents, at various equivalents per monomer residue, by using previously reported procedures.[38,39] Such modified PEIs have been used as enzyme models ("synzymes"), suggesting that the modifications can substantially change their molecular recognition properties.[40,41] Derivatization carried out in this way has been shown to change the hydrophobicities of these compounds, as manifested in, for example, altered pK_a values of polymer amines.[38,39] The various quantities of derivatization reagents allow quantitative definition of chemical space (represented by the *x*-, *y*-, and *z*-axes of methyl, benzyl, and dodecyl derivatization equivalents in Figure 1). The resulting collection of 435 compounds smoothly samples the ensuing diversity. The modifications (Scheme 1) introduce positive charge by quaternization of amine groups[35] (to bind DNA) and at the same time

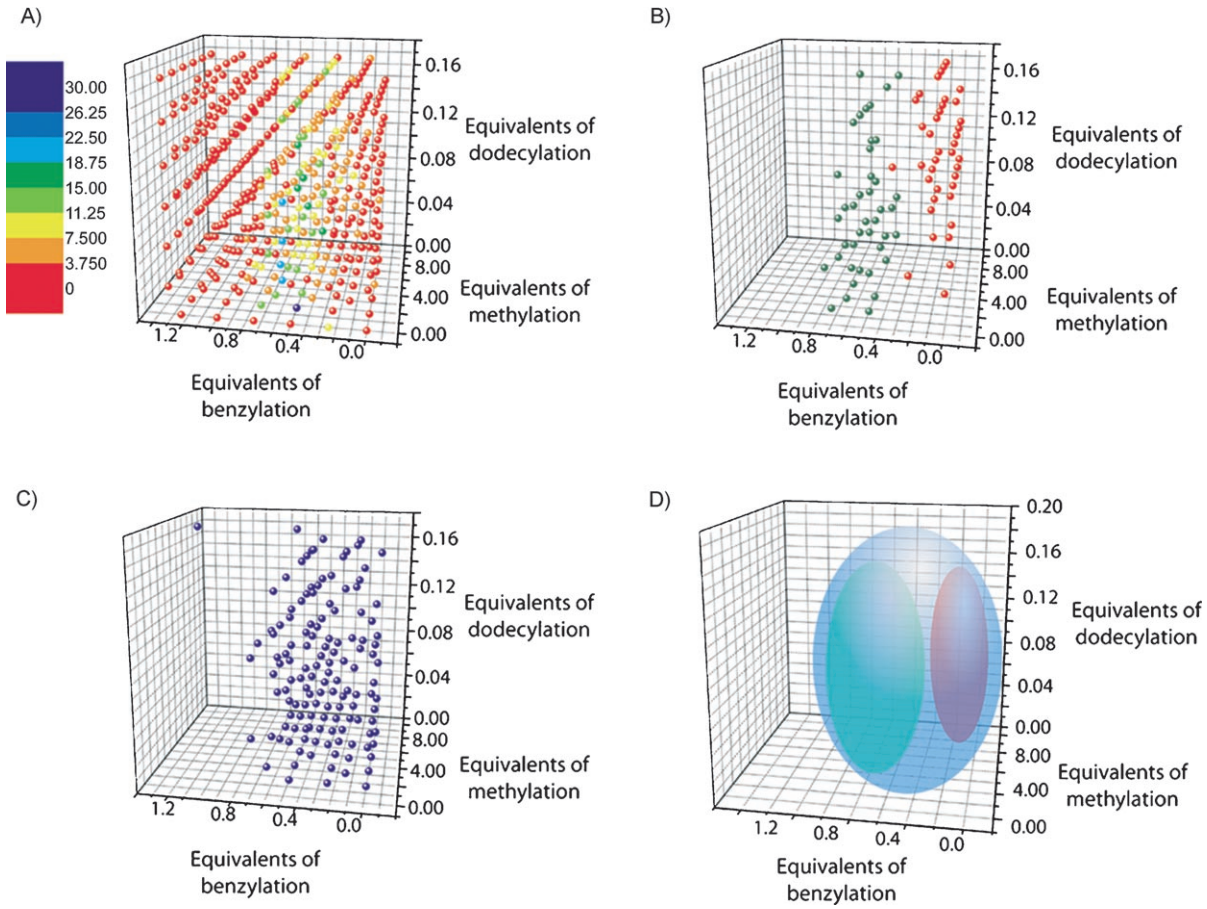
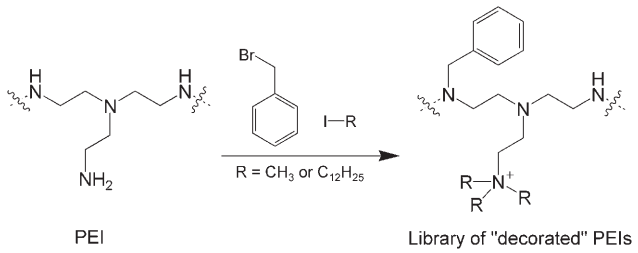


Figure 1. Our library displays clear relationships between derivatization and activity. A–D) Functional space maps into chemical derivatization space, by displaying transfection, DNA binding, and toxicity data as a function of the amount of derivatization reagents applied (in equivalents of reagent per PEI amine). A) Transfection data for the *entire* library. Data points are colored in proportion to the number of cells that expressed EGPF when that polymer was used in the transfection assay; this panel is displayed from different angles in Figure S5. B) The best transfection reagents and the most toxic polymers (i.e., polymers that lead to cell membrane damage measured by LDH leakage) appear in distinct, defined areas. Polymers defined as good transfection reagents (●): $\geq$10% of cells expressed EGFP and $\leq$5% stained with propidium iodide after transfection; polymers with high toxicity (●); >5% cells were stained with propidium iodide after transfection. C) How polymers with improved DNA binding ($F_r \leq 0.3$) map into a defined area. D) Combined information from B) and C) in a cartoon representation: the area of high transfection is green, that of high toxicity is red, and both these areas are encompassed by the area of strong DNA binding in blue. The complete data for transfection, toxicity, and DNA binding are shown in Table S1. Transfection conditions: 0.25 μg pEGFP-C1 per well, N:P ratio = 10.



Scheme 1. Derivatization of PEI by using simple alkyl substitution chemistry to produce diverse libraries. PEI25 was treated with different amounts of each reagent that react with primary, secondary, and tertiary amines to create a range of products, of which one representative example is shown.

alter the hydrophobic character of the polymer[39] (aiding membrane penetration of the PEI–DNA complex).

We tested this library using three high-throughput assays (for overall transfection, DNA binding, and toxicity) and were able to visualize the synergistic effect of combining multiple derivatizations as a function of these assays. This approach provided a comprehensive illustration of mapping functional data into chemical space.[37]

Results and Discussion

To define the functional consequences of PEI derivatization, we tested our library in three robust, high-throughput assays carried out in microwell formats that profiled the effects of small changes on the physical and chemical properties of the polymer library at different stages of the transfection process.

1) To study transfection efficiency, a plasmid (pEGFP-C1) encoding enhanced green fluorescent protein (EGFP) was used as a reporter gene with the CHO-K1 cell line. Using a fluorescent reporter gene allows measurement both of the total level of protein expression and of the percentage of cells expressing the reporter. Here we have chosen to use the percentage of cells showing expression as our measure of transfection (Figure S9 in the Supporting Information).[42]
2) The toxicity of the PEI transfection reagents was assessed at the same time as transfection efficiency by counting the number of nonviable cells stained with propidium iodide[43] and the total number of cells.
3) Finally, the ability of the derivatized polymers to bind and compact DNA was measured by an ethidium displacement assay.[44] To compare large numbers of compounds an equal amount of each polymer was added to a standard solution of DNA and ethidium bromide, and the decrease in fluorescence was compared in order to give a relative fluorescence value (F_r).

Transfection (1) and toxicity (2) assays were carried out in triplicate in three separate experiments. The entire dataset for 435 polymers thus obtained represents the readout of >3900 transfection measurements. Figure 1 correlates these data points with >1300 DNA binding assays (for 435 polymers performed in triplicate) and comprises the data of more than 5200 separate experiments in total (listed in Table S1).

Functional diversity across the derivatization space

Although the region of chemical space sampled by this polymer collection is defined by only three building blocks, resulting in a low hypothetical formal diversity score,[45] the results from these assays display a remarkable amount of actual functional diversity (Figure 2). The most toxic polymer (3-F5, derivatized with 4.3 equiv methyl iodide, 0.13 equiv benzyl bromide, and 0.14 equiv dodecyl iodide) is over 50-times more toxic than the unmodified PEI, while 15 members of the library are slightly less toxic than PEI. The largest observed difference in transfection efficiency between the best and worst PEI derivatives is 100-fold. The most efficient transfection reagent (1-C3, derivatized with 1.7 equiv methyl iodide and 0.5 equiv benzyl bromide) is over 80-times more efficient than underivatized PEI.[46] The best compounds (shown in Table 1) rival the commercial reagents JetPEI™ and Lipofectamine™ 2000.

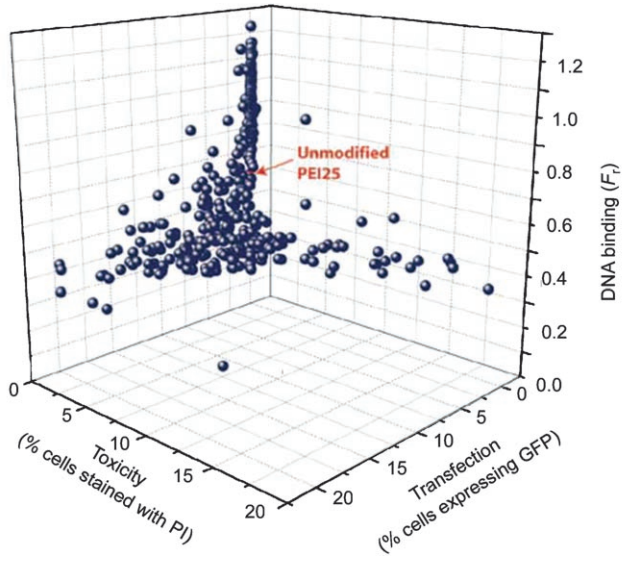


Figure 2. Limited derivatization building blocks create polymers with diverse properties. Transfection efficiency, toxicity, and DNA binding data are plotted for all 435 derivatized PEIs, with the underivatized polymer shown in red and marked with an arrow. Transfection conditions: 0.25 μg of pEGFP was used per well of a 96-well plate, complexed with derivatized PEI at a N:P ratio of 10:1. Note that the measure of relative DNA binding (F_r) decreases as binding affinity increases.

Overall, 24% of all polymers showed a greater than fivefold increase in EGFP expression over the unmodified polymer. The high occurrence of improved candidates amongst the PEI derivatives suggests that the properties altered by our substituents play a key role in the transfection process. Along with high diversity, the properties of this library show clear relationships to derivatization, such as that between EGFP expression and derivatization in Figure 1 A. To visualize the comparison of this relationship with DNA binding and toxicity assays, reagents were classified into groups by their activity in each of

Table 1. The best derivatized PEIs rival commercial transfection reagents in transfection activity. Transfection, toxicity, and DNA binding results are shown for the commercial reagents Lipofectamine™ 2000, JetPEI™, our best derivatized PEIs, and unmodified 25 kDa PEI (PEI25).[a]

Reagent	Transfection [%][b]	Toxicity [%][b]	DNA binding [F_r][c]
JetPEI	31.5 ± 5.0	0.32 ± 0.03	0.09 ± 0.11
Lipofectamine 2000	21.2 ± 10.3	0.16 ± 0.10	N/A
PEI25	0.33 ± 0.24	0.13 ± 0.13	0.50 ± 0.08
1-B4	19.3 ± 6.8	2.7 ± 3.2	0.26 ± 0.06
1-C3	28.4 ± 7.4	2.0 ± 2.7	0.21 ± 0.05
2-B4	20.3 ± 3.9	1.0 ± 0.8	0.32 ± 0.03

[a] Values are means of n experiments ($n = 9$ for transfection and toxicity, $n = 4$ for DNA binding) ± standard error. Transfection conditions: 0.25 μg of pEGFP was used per well of a 96-well plate, together with derivatized PEI, PEI25, or JetPEI™ at an N:P ratio of 10:1. Lipofectamine™ 2000 was used with 2 μg pEGFP per well. [b] Transfection and toxicity results show percentages of cells that expressed EGFP or stained with propidium iodide after transfection in CHO-K1 cells. Values for toxicity are likely to be a lower limit as some dead cells can be removed in a washing step (see the Experimental Section). [c] The DNA binding column gives relative fluorescence (F_r), which decreases with increased affinity for DNA.

the three assays. Figure 1B and 1C show the top 10% of PEI derivatives in terms of transfection efficacy, toxicity (Figure 1B), and DNA binding (Figure 1C). The groups of good transfection reagents and toxic polymers (Figure 1B) do not overlap. The group of strong DNA binders encompasses the former two. For easy reference, the relationships between these three functions are combined as a cartoon (Figure 1D). This diagram represents all biological data as a function of the in situ derivatization of PEI: that is, is the projection of "biological" descriptor space into "chemical" derivatization space.

Characterization of polymer–DNA interactions

Our derivatizations alter the charges of the polymers and so might affect the ratio of polymer amines to DNA phosphates (N:P ratio) required to form a successful transfection complex. One possible explanation for the clear band of good transfection reagents running through our chemical derivatization space in Figure 1B is that these were the polymers the charges of which were tuned to the N:P ratio of 10:1 used in the standard assays. To investigate the effect of changing this ratio, compounds were tested with N:P ratios of 5, 10, 15, and 20:1 (Figure S4). Although the overall levels of transfection and toxicity varied, the functional relationships did not significantly alter from those seen with an N:P ratio of 10:1. This shows that they are inherent properties of the reagents, and not the result of tuning the charge of the polymer to the N:P ratio chosen for the experiment.

We also addressed the question of whether the effects seen were due to modifications found on one particular polymer and not just a formulation of different molecules with various properties. To this end we measured transfection for a mixture of singly modified polymers at a ratio that matched the same overall composition of good transfection reagents; that is, PEI (*y* equiv benzylation; *x* equiv methylation) was compared to PEI (*y* equiv benzylation) mixed with PEI (*x* equiv methylation). For example, PEI derivatized with 0.4 equiv of methylation and 0.9 equiv of benzylation gave 25-times more EGFP expression than when PEI derivatized with 0.4 equiv of methyl iodide was mixed with PEI derivatized with 0.9 equiv of benzyl bromide (data not shown). Generally the transfection efficiency was reduced by 10–25-fold; this indicates that mounting the functionality on one polymer framework is important.

Cell-type selectivity

For possible in vivo cell targeting, selectivity of transfection reagents can be of great utility. We addressed this possibility using two model cell lines: CHO-K1 and Ishikawa cells. Unmodified PEI exhibited little difference in transfection efficiency between CHO-K1 and Ishikawa cells (3.4 ± 1.2 and 5.3 ± 0.7% of cells transfected, respectively). Across the three-dimensional chemical space (Figure 1D) they showed an overall similar profile, but slight shifts can be exploited to identify individual compounds within the area of high transfection that discriminate for particular cell types. For example, polymer 5-C2 (derivatizations given in Table S1) is more tuned to the requirements of CHO-K1 cells (7.0 ± 2.9% transfected) than Ishikawa cells (0.7 ± 0.5% transfected); this corresponds to a tenfold discrimination. Polymer 2-H6, however, is tuned in the opposite direction: 1.3 ± 0.1% transfection in CHO-K1, but 8.2 ± 4.1% in Ishikawa cells. These data suggest that it is possible to select reagents that are more finely tuned to one cell over another within the chemical space resulting from PEI derivatization.

Discussion

Previous work to improve polymeric nonviral transfection has been based on individual modifications to the polymer[24, 25, 34, 35, 47–49] or on changes in backbone properties.[15, 26–29] We now demonstrate that surface modification of PEI can further improve PEI transfection by up to two orders of magnitude. We have combined up to three types of derivatizations and have found that our best compounds require a combination of modifications, acting synergistically to increase transfection efficiency by roughly an order of magnitude more than the sum of each single modification.

By comparing results from different assays, we can begin to understand how these interrelate with the mechanism of transfection. All of the compounds that show strong biological activity, whether in transfection or toxicity, demonstrate increased DNA binding relative to the unmodified polymer (i.e., the blue DNA-binding region in Figure 1D that encompasses the transfection and toxicity regions). Since the first stage in successful transfection is the formation of a PEI–DNA complex, the fact that all of the most active transfection reagents have strong affinities for DNA is to be expected. On the other hand, PEI toxicity might be due either to binding to cellular nucleic acids[50] or to membrane damage.[51] Free PEI that has entered the cell independently or as part of a complex might bind to genomic DNA and interfere with normal cellular functions. This type of toxicity is dependent on the affinity of PEI for DNA.

The second form of PEI toxicity is due to damage to membranes, including endosomal membranes. Endosomal membrane damage depends on the buffering capacity of the polymer,[52] which is in turn a function of its level of derivatization.[53] Figure 1 D shows that while all of the polymers with significant toxicity have a greater affinity for DNA than unmodified PEI, many that show good DNA binding also have low toxicity. This finding is reinforced by a plot of DNA binding against toxicity and transfection (Figure 3), in which the levels of DNA binding are similar for the different members of our library that show high toxicity or alternatively high transfection. For example, of the two best DNA binders (3-F5 and 1-C3), 3-F5 is a mediocre transfection reagent (0.4% transfected cells) and 1-C3 is the best (28.4% transfected cells). These observations suggest that the ability to bind DNA is necessary but not sufficient for toxicity or transfection and that another property—buffering capacity or DNA release kinetics, for example—might also contribute. While increased DNA binding is necessary for activity, the regions characterized by high transfection or toxicity (Figure 1 B, green and red) do not overlap; this suggests that high transfection efficiency is compromised by toxicity.

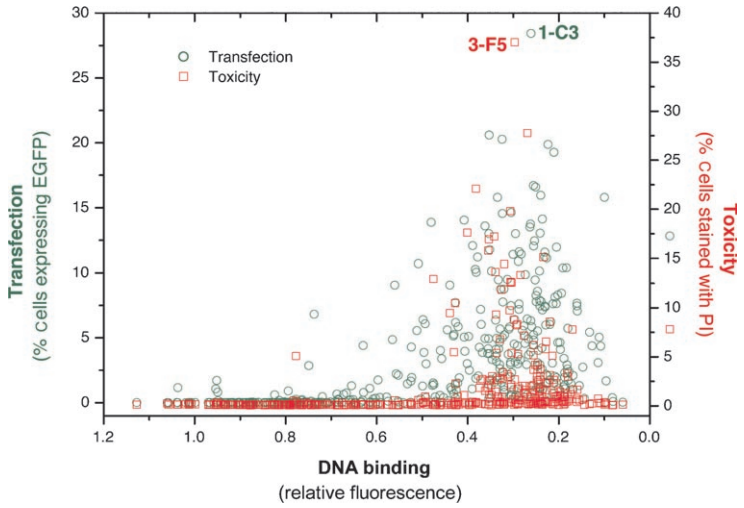


Figure 3. DNA binding is important, but not the sole determinant of biological activity. Each data point in this plot represents the functional characteristics of one member of our library. Stronger DNA binding (on the right hand side) can lead either to high transfection or to high toxicity—or even to no special activity at all. For example, good DNA binding (represented by $F_r \sim 0.26$) leads to high transfection for polymer 1-C3, but similar DNA binding can also lead to high toxicity in 3-F5 (and low transfection activity). In addition, there are many polymers in which comparable DNA binding leads to little or no biological activity. This means that DNA binding is a necessary, but not sufficient, condition for biological activity of PEI derivatives and that as such it does not alone determine whether a polymer is toxic or a good transfection reagent.

While the role of the polymer backbone has been extensively studied with large libraries of several hundred members,[15,26,28,33,35,54] modification of the peripheries of small-molecule scaffolds (e.g., gemini surfactants) has involved relatively small compound collections (typically <20 members).[15,25,35,49] The small sizes of the compound collections as well as the complexity of the transfection process have made it difficult to observe structure–activity relationships as a function of the derivatization reagents. The larger sizes of polymer backbone libraries have resulted in large improvements in activity,[15,26,28,33,35,54] but it has been difficult to visualize any structure–activity relationship because defining the axes that represent chemical diversity space (as in Figure 1) is not obvious.

Although we have only derivatized PEI with three substituents of limited chemical functionality, using straightforward chemistry, our library displays a diverse range of properties that are systematically related (Figure 2), including differences of one to two orders of magnitude in both toxicity and transfection. More diverse appended building blocks and previously discovered alternative polymer backbones[15,26,28] in combination with our "polymer decoration" approach could provide a route towards more efficient transfection reagents.

Any combinatorial experiment is a compromise between wider coverage and intensive mapping of a focused area of diversity space.[37] Our results suggest that the transfection activity of PEI is sufficiently sensitive to modification that complete sampling of a smaller fraction of derivatization space generates a high degree of *functional* diversity, despite the very limited *chemical* diversity of the building blocks used. The effect of polymer modification is thus much larger than previously demonstrated or estimated with diversity assessments.[45] By contrast, the degrees of freedom for modifying small molecules are much more limited, not only by the synthetic chemistry, but also by the limited number of atoms that can be changed or modified. A multivalent polymer not only allows greater numbers of modifications, but the proportions of these building blocks can be used to fine-tune the properties (e.g., the ratio between hydrophobic and charged groups) with greater sensitivity, and to define diversity as a continuous function of derivatization. This is difficult or impossible for compound collections that are not amenable to regular derivatization patterns as PEI is. If, as we demonstrate in the case of transfection, the activity "peak regions" in chemical diversity space are narrow bands rather than large areas (Figure 1), then the complete sampling of this diversity space is crucial, and the versatility of a multimeric framework that can be functionalized with varying reagent combinations is more likely to succeed. We suspect that this conclusion might apply more generally to the synthesis of drug delivery formulations and reagents.

Conclusions

A key contribution of chemistry to biology is to vary chemical structure systematically to improve the biological activity of a given molecule and to create new function. Despite a great deal of research effort, this approach has achieved only limited success for the central biological challenge of delivering nucleic acids to mammalian cells with chemical vectors. Modifica-

tions of the known transfection reagent polyethyleneimine (PEI) make its efficiency similar to those of commercial reagents, despite the limited functional diversity of the derivatization building blocks. This suggests that polymer modification can enhance existing transfection reagents substantially. The success of this approach involves a strategy that starts with the creation of a library of PEI derivatives that extensively sample a three-dimensional region of chemical space, taking advantage of the polymer's multivalency and potential for chemical decoration, followed by multidimensional biological assays. In this case, a systematic screen of the library to assess the degree of functional diversity in three high-throughput assays for properties relevant to successful transfection (toxicity, DNA binding, and overall transfection efficiency) revealed clear relationships within this chemical space. This work demonstrates that the modification of a polymer framework produced much greater diversity than previously believed, leading us to rethink established ideas about the inherent compromise between coverage and density of sampling in combinatorial experiments. Conventional wisdom is to create large libraries that cover a wide variety of chemical modifications. Instead, we found improvements by densely sampling one region of chemical space.

In addition to large improvements in activity, some of our reagents showed cell type specificity, indicating that we can tune supramolecular properties to the particular requirements of transfection in a specific cell line that cannot be predicted, thus validating the combinatorial approach. By combining the results of the multidimensional relationships in chemical space (conveniently defined by derivatization quantities), we provide a basis for understanding the underlying mechanisms. We believe that this novel approach to an important problem provides a system for tuning transfection reagents and in addition provides insight into the requirements of gene delivery. More generally, it might be possible to apply this systematic approach to other cases in which it is necessary to screen large chemical libraries for a particular biological function.

Experimental Section

Materials: All chemical reagents (including PEI) were purchased from Sigma–Aldrich. Media, Nonessential amino acids (NEAA), Opti-MEM 1®, and antibiotics were purchased from Gibco (Paisley, UK). HEPES-buffered saline (HBS) contained 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (1 mM) and sodium chloride (150 mM) with the pH adjusted to 7.4 by addition of aqueous sodium hydroxide solution (1 M).

Library synthesis: A solution of PEI (25 kDa, 0.317 M in monomer residues) in *N*,*N*-dimethylformamide (DMF, 458.9 mL) and 2,6-lutidine (5.85 mL) was added (0.9 mL) into deep-well 96-well plates. Solutions of benzyl bromide and lauryl iodide (175 μL, various concentrations) in DMF were added to each well to give the required combinations, and the reaction mixtures were stirred with magnetic stirring bars for five days at room temperature. Various concentrations of methyl iodide (350 μL, various concentrations) in DMF were added, followed by a further three days of stirring at room temperature. An overview of the synthesis is shown in Scheme 1. Mass spectra of incubations of hexylamine with the three reagents used in this study show that the starting material has been effectively turned over on the timescale employed for derivatization (Supporting Information). An assay of primary amines in PEI (25 kDa) by treatment with ninhydrin[55] shows that the derivatization also occurs efficiently on polymer amines. These data suggest that the high reactivity of the reagents is sufficient to derivatize the polymer in the time allowed in this procedure.

To hydrolyze any unreacted alkylating reagents, a sample from each well (15 μL) was transferred to another 96-well plate, where it was diluted with DMF (135 μL) and NaOH (1350 μL, 50 mM) and incubated, overnight, with shaking. The derivatization ratios of each library member are shown in Table S1, together with the functional data obtained for each of them. Reaction products that were resynthesized on a larger scale and purified by dialysis[38–40] maintain their activities (within error); this suggests that the activities observed are indeed a function of the derivatized PEI. Modifications with larger derivatization ratios than shown in Figure 1 appeared not to change transfection properties further.

Ethidium displacement assay: Samples of each hydrolyzed compound (30 μL) were further diluted in HBS (166 μL) and acetic acid (4 μL, 87.5 mM). The diluted compounds (20 μL) were added to the wells of a 96-well plate containing calf thymus DNA (3 μL pUC19, 0.25 mM), ethidium bromide (5 μL, 0.25 mM), and HBS (to 200 μL). The ethidium fluorescence of each well containing a polymer and of wells without polymer or DNA was measured (BMG FluoroStar Optima, $\lambda_{ex}=260$ nm and $\lambda_{em}=595$ nm), and relative fluorescence (F_r) was calculated according to Equation (1):

$$F_r = (F_{obs}-F_e)/(F_0-F_e) \quad (1)$$

where F_{obs} is the observed fluorescence of the sample, F_0 the fluorescence in the absence of PEI, and F_e the background fluorescence in the absence of DNA.

Cell culture: Chinese hamster ovary cells (CHO-K1) were cultured in F12 (Ham) medium with fetal bovine serum (10%), glutamine, and penicillin/streptomycin. Ishikawa cells were cultured in D-MEM medium with fetal bovine serum (10%), NEAA (1%), glutamine, and penicillin/streptomycin. Cells were seeded into 96-well plates 18 h before transfection at a density of 20000 cells per well.

Transfection and toxicity: The structures and properties of PEI–DNA complexes depend strongly on the ratio of polymer amine groups to DNA phosphates (N:P ratio).[16] For our initial experiments we chose an N:P ratio of 10:1, which is close to the optimum for unmodified PEI.[16] Derivatized PEI or JetPEI (20 μL, 0.37 μM in amine residues) or lipofectamine 2000 (20 μL, 0.2 mg mL^{-1}) was mixed with an equal volume of pEGFP-C1 (20 μL, 37 μM phosphate) to give the correct N:P ratio. After incubation (30 min, 20 °C) to allow complex formation, the complex was diluted with Opti-ME M1® (160 μL) and added (100 μL) to wells originally filled with 20000 cells. After incubation with the transfection mixture (5 h, 37 °C), cells were washed with PBS and grown overnight. Plates were washed with phosphate-buffered saline (PBS) before addition of trypsin (30 μL) and incubation (5 min, 37 °C). Each well of cells was diluted in PBS (240 μL), stained with propidium iodide (30 μL, 10 μg mL^{-1}, 20 min), and read with a Beckman–Coulter FC500 MPL flow cytometer. Each compound was tested in triplicate in three separate experiments to give nine replicates, from which the mean and standard error were calculated. Resynthesis of PEI derivatives reproduced the same activity patterns and levels. Under the conditions employed in this work the average transfection efficiency for underivatized PEI was 0.33% ± 0.24. A larger level of transfection for underivatized PEI has been reported

under a different set of conditions (i.e., larger amounts of DNA: up to 1.5 μg for 20000 cells in previous work[28,56] instead of 0.25 μg per well of 20000 cells here). However, we chose the latter set of conditions to be able to represent improvements within the dynamic range of the assay and to differentiate better between underivatized PEI and its derivatives (Figure S7). Furthermore, we found the transfection activity of *underivatized* commercial PEI to show a batch-to batch variation of approximately fivefold. However, this variation did not extend to PEI derivatives. All assays reproducibly identified identical frontrunners and relative activities of the library members within the errors given in Table S1.

We monitored the total cell count after transfection by flow cytometry to rule out any possibility that a washing step included in the procedure would bias the data because of possible removal of dead cells before the toxicity assay. However, the total number of cells in all but a very few wells (<5%) remains within error (Figure S6). Those wells in which the cell count was lowered (up to 20% below average) were randomly distributed and did not correlate to the cells with low toxicity measured by the propidium iodide assay, suggesting that a systematic bias by the washing step can safely be excluded.[57]

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Keywords: chemical space · combinatorial chemistry · gene technology · nonviral gene delivery · polyethyleneimine

[1] L. Cao, Z. C. Zheng, Y. C. Zhao, Z. H. Jiang, Z. G. Liu, S. D. Chen, C. F. Zhou, X. Y. Liu, *Hum. Gene Ther.* **1995**, *6*, 1497–1501.
[2] I. M. Verma, M. D. Weitzman, *Annu. Rev. Biochem.* **2005**, *74*, 711–738.
[3] a) T. Niidome, L. Huang, *Gene Ther.* **2002**, *9*, 1647–1652; b) J. A. Wolff, *Nat. Biotechnol.* **2002**, *20*, 768–769.
[4] M. E. Davis, *Curr. Opin. Biotechnol.* **2002**, *13*, 128–131.
[5] M. Thomas, A. M. Klibanov, *Appl. Microbiol. Biotechnol.* **2003**, *62*, 27–34.
[6] a) W. H. Sun, J. K. Burkholder, J. Sun, J. Culp, J. Turner, X. G. Lu, T. D. Pugh, W. B. Ershler, N. S. Yang, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 2889–2893; b) H. Herweijer, J. A. Wolff, *Gene Ther.* **2007**, *14*, 99–107.
[7] J. Dobson, *Gene Ther.* **2006**, *13*, 283–287.
[8] a) C. Chittimalla, L. Zammut-Italiano, G. Zuber, J. P. Behr, *J. Am. Chem. Soc.* **2005**, *127*, 11436–11441; b) K. Na, S. Kim, K. Park, K. Kim, D. G. Woo, I. C. Kwon, H. M. Chung, K. H. Park, *J. Am. Chem. Soc.* **2007**, *129*, 5788–5789; c) C. R. Safinya, *Curr. Opin. Struct. Biol.* **2001**, *11*, 440–448.
[9] a) G. T. Zugates, D. G. Anderson, S. R. Little, I. E. Lawhorn, R. Langer, *J. Am. Chem. Soc.* **2006**, *128*, 12726–12734; b) M. D. Brown, A. G. Schatzlein, I. F. Uchegbu, *Int. J. Pharm.* **2001**, *229*, 1–21; c) H. Eliyahu, Y. Barenholz, A. J. Domb, *Molecules* **2005**, *10*, 34–64; d) T. Merdan, J. Kopecek, T. Kissel, *Adv. Drug Delivery Rev.* **2002**, *54*, 715–758; e) D. W. Pack, A. S. Hoffman, S. Pun, P. S. Stayton, *Nat. Rev. Drug Discovery* **2005**, *4*, 581–593; f) T. Segura, L. D. Shea, *Ann. Rev. Mater. Res.* **2001**, *31*, 25–46; g) J. A. Wolff, D. B. Rozema, *Mol. Ther.* **2007**, *16*, 8–15.
[10] A. J. Kirby, P. Camilleri, J. B. Engberts, M. C. Feiters, R. J. Nolte, O. Soderman, M. Bergsma, P. C. Bell, M. L. Fielden, C. L. Garcia Rodriguez, P. Guedat, A. Kremer, C. McGregor, C. Perrin, G. Ronsin, M. C. van Eijk, *Angew. Chem.* **2003**, *115*, 1486–1496; *Angew. Chem. Int. Ed.* **2003**, *42*, 1448–1457.
[11] C. R. Safinya, K. Ewert, A. Ahmad, H. M. Evans, U. Raviv, D. J. Needleman, A. J. Lin, N. L. Slack, C. George, C. E. Samuel, *Philos. Trans. R. Soc. London Ser. A* **2006**, *364*, 2573–2596.
[12] I. van der Woude, A. Wagenaar, A. A. Meekel, M. B. ter Beest, M. H. Ruiters, J. B. Engberts, D. Hoekstra, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 1160–1165.
[13] C. W. Pouton, P. Lucas, B. J. Thomas, A. N. Uduehi, D. A. Milroy, S. H. Moss, *J. Controlled Release* **1998**, *53*, 289–299.
[14] a) J. Haensler, F. C. Szoka, Jr., *Bioconjug. Chem.* **1993**, *4*, 372–379; b) D. R. Radu, C. Y. Lai, K. Jeftinija, E. W. Rowe, S. Jeftinija, V. S. Lin, *J. Am. Chem. Soc.* **2004**, *126*, 13216–13217.
[15] M. Krämer, J. F. Stumbé, G. Grimm, B. Kaufmann, U. Krüger, M. Weber, R. Haag, *ChemBioChem* **2004**, *5*, 1081–1087.
[16] O. Boussif, F. Lezoualch, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, J. P. Behr, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 7297–7301.
[17] a) J.-S. Remy, B. Abdallah, M. A. Zanta, O. Boussif, J.-P. Behr, B. Demeneix, *Adv. Drug Delivery Rev.* **1998**, *30*, 85–95; b) M. Neu, D. Fischer, T. Kissel, *J. Gene Med.* **2005**, *7*, 992–1009.
[18] A. D. Miller, *Angew. Chem.* **1998**, *110*, 1862–1880; *Angew. Chem. Int. Ed.* **1998**, *37*, 1768–1785.
[19] K. Ewert, H. M. Evans, A. Ahmad, N. L. Slack, A. J. Lin, A. Martin-Herranz, C. R. Safinya, *Adv. Genet.* **2005**, *53PA*, 119–155.
[20] I. S. Zuhorn, J. B. Engberts, D. Hoekstra, *Eur. Biophys. J.* **2007**, *36*, 349–362.
[21] B. Ma, S. Zhang, H. Jiang, B. Zhao, H. Lv, *J. Control. Release* **2007**, *123*, 184–194.
[22] C. McGregor, Ph.D. thesis, University of Cambridge, **2001**.
[23] M. Scarzello, J. Smisterova, A. Wagenaar, M. C. Stuart, D. Hoekstra, J. B. Engberts, R. Hulst, *J. Am. Chem. Soc.* **2005**, *127*, 10420–10429.
[24] S. R. Popielarski, S. Mishra, M. E. Davis, *Bioconjugate Chem.* **2003**, *14*, 672–678.
[25] K. Lenssen, P. Jantscheff, G. von Kiedrowski, U. Massing, *ChemBioChem* **2002**, *3*, 852–858.
[26] D. G. Anderson, W. Peng, A. Akinc, N. Hossain, A. Kohn, R. Padera, R. Langer, J. A. Sawicki, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 16028–16033.
[27] a) D. M. Lynn, D. G. Anderson, D. Putnam, R. Langer, *J. Am. Chem. Soc.* **2001**, *123*, 8155–8156; b) D. G. Anderson, D. M. Lynn, R. Langer, *Angew. Chem.* **2003**, *115*, 3261–3266; *Angew. Chem. Int. Ed.* **2003**, *42*, 3153–3158.
[28] A. Akinc, D. M. Lynn, D. G. Anderson, R. Langer, *J. Am. Chem. Soc.* **2003**, *125*, 5316–5323.
[29] a) D. G. Anderson, A. Akinc, N. Hossain, R. Langer, *Mol. Ther.* **2005**, *11*, 426–434; b) A. N. Zelikin, D. Putnam, P. Shastri, R. Langer, V. A. Izumrudov, *Bioconjugate Chem.* **2002**, *13*, 548–553.
[30] J. J. Green, J. Shi, E. Chiu, E. S. Leshchiner, R. Langer, D. G. Anderson, *Bioconjugate Chem.* **2006**, *17*, 1162–1169.
[31] G. T. Zugates, W. Peng, A. Zumbuehl, S. Jhunjhunwala, Y. H. Huang, R. Langer, J. A. Sawicki, D. G. Anderson, *Mol. Ther.* **2007**, *15*, 1306–1312.
[32] a) M. Mammen, S. K. Choi, G. M. Whitesides, *Angew. Chem.* **1998**, *110*, 2908–2953; *Angew. Chem. Int. Ed.* **1998**, *37*, 2754–2794; b) R. Haag, F. Kratz, *Angew. Chem.* **2006**, *118*, 1218–1237; *Angew. Chem. Int. Ed.* **2006**, *45*, 1198–1215.
[33] M. Thomas, J. J. Lu, Q. Ge, C. Zhang, J. Chen, A. M. Klibanov, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 5679–5684.
[34] N. P. Gabrielson, D. W. Pack, *Biomacromolecules* **2006**, *7*, 2427–2435.
[35] M. Thomas, A. M. Klibanov, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 14640–14645.
[36] M. Thomas, Q. Ge, J. J. Lu, J. Chen, A. M. Klibanov, *Pharm. Res.* **2005**, *22*, 373–380.
[37] a) S. J. Haggarty, *Curr. Opin. Chem. Biol.* **2005**, *9*, 296–303; b) C. M. Dobson, *Nature* **2004**, *432*, 824–828.
[38] F. Hollfelder, A. J. Kirby, D. S. Tawfik, *J. Am. Chem. Soc.* **1997**, *119*, 9578–9579.
[39] F. Hollfelder, A. J. Kirby, D. S. Tawfik, *J. Org. Chem.* **2001**, *66*, 5866–5874.
[40] F. Avenier, J. B. Domingos, L. D. van Vliet, F. Hollfelder, *J. Am. Chem. Soc.* **2007**, *129*, 7611–7619.
[41] a) J. Suh, *Synlett* **2001**, 1343–1363; b) L. Liu, R. Breslow, *J. Am. Chem. Soc.* **2002**, *124*, 4978–4979; c) L. Liu, W. Zhou, J. Chruma, R. Breslow, *J. Am. Chem. Soc.* **2004**, *126*, 8136–8137; d) I. Klotz in *Enzyme Mechanisms*

(Eds.: M. I. Page, A. Williams), Royal Society of Chemistry, London, **1987**, pp. 14–34; e) I. M. Klotz, J. Suh in *Artificial Enzymes* (Ed.: R. Breslow), Wiley-VCH, Weinheim, **2005**, pp. 63–88.

[42] Figure S9 in the Supporting Information shows a comparison between the percentage of cells that express EGFP and total fluorescence intensity (used frequently in other studies). These parameters are linearly related for the set of conditions used in this study.

[43] I. Nicoletti, *J. Immunol. Methods* **1991**, *139*, 271–279.

[44] B. F. Cain, B. C. Baguley, W. A. Denny, *J. Med. Chem.* **1978**, *21*, 658–668.

[45] a) S. Fergus, A. Bender, D. R. Spring, *Curr. Opin. Chem. Biol.* **2005**, *9*, 304–309; b) J. J. Perez, *Chem. Soc. Rev.* **2005**, *34*, 143–152.

[46] A comparison to transfection by DNA alone has been used to evaluate transfection reagents.[30] However, the DNA at the concentration used in this study does not transfect significantly.

[47] P. C. Bell, M. Bergsma, I. P. Dolbnya, W. Bras, M. C. Stuart, A. E. Rowan, M. C. Feiters, J. B. Engberts, *J. Am. Chem. Soc.* **2003**, *125*, 1551–1558.

[48] R. Smolarczyk, T. Cichon, A. Sochanik, S. Szala, *Cytokine* **2005**, *29*, 283–287.

[49] C. McGregor, C. Perrin, M. Monck, P. Camilleri, A. J. Kirby, *J. Am. Chem. Soc.* **2001**, *123*, 6215–6220.

[50] M. Thomas, A. M. Klibanov, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 9138–9143.

[51] S. M. Moghimi, P. Symonds, J. C. Murray, A. C. Hunter, G. Debska, A. Szewczyk, *Mol. Ther.* **2005**, *11*, 990–995.

[52] N. D. Sonawane, F. C. Szoka, Jr., A. S. Verkman, *J. Biol. Chem.* **2003**, *278*, 44826–44831.

[53] A. Akinc, M. Thomas, A. M. Klibanov, R. Langer, *J. Gene Med.* **2005**, *7*, 657–663.

[54] a) C. M. Varga, N. C. Tedford, M. Thomas, A. M. Klibanov, L. G. Griffith, D. A. Lauffenburger, *Gene Ther.* **2005**, *12*, 1023–1032; b) D. H. Wakefield, J. J. Klein, J. A. Wolff, D. B. Rozema, *Bioconjugate Chem.* **2005**, *16*, 1204–1208.

[55] H. Tian, W. Xiong, J. Wei, Y. Wang, X. Chen, X. Jing, Q. Zhu, *Biomaterials* **2007**, *28*, 2899–2907.

[56] a) C. L. Gebhart, A. V. Kabanov, *J. Controlled Release* **2001**, *73*, 401–416; b) W. T. Godbey, A. G. Mikos, *J. Controlled Release* **2001**, *72*, 115–125; c) L. Poulain, C. Ziller, C. D. Muller, P. Erbacher, T. Bettinger, J. F. Rodier, J. P. Behr, *Cancer Gene Ther.* **2000**, *7*, 644–652; d) Q. R. Chen, L. Zhang, P. W. Luther, A. J. Mixson, *Nucleic Acids Res.* **2002**, *30*, 1338–1345; e) M. L. Read, S. Singh, Z. Ahmed, M. Stevenson, S. S. Briggs, D. Oupicky, L. B. Barrett, R. Spice, M. Kendall, M. Berry, J. A. Preece, A. Logan, L. W. Seymour, *Nucleic Acids Res.* **2005**, *33*, e86; f) J. P. Clamme, G. Krishnamoorthy, Y. Mely, *Biochim. Biophys. Acta Biomembr.* **2003**, *1617*, 52–61; g) W. Dong, S. Li, G. Jin, Q. Sun, D. Ma, Z. Hua, *Int. J. Mol. Sci.* **2007**, *8*, 81–102.

[57] Propidium iodide stained cells that have survived a washing step could have internal damage that leads to a "long-term" (overnight) effect of a particular compound that has entered the cell. This common toxicity measure merely describes the amount of damage to cells that die as the transfection reagents enter the cell. After overnight incubation these cells are no longer viable, and would be washed away. To account for this, we measured the total count of cells per unit per well.

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