Quantitation of Complexed versus Free Polymers in Interpolyelectrolyte Polyplex Formulations

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S *Supporting Information*

ABSTRACT: The quantity of free polymer in a polymer/DNA complex (polyplex) formulation critically impacts its gene transfection efficiency, cellular uptake, and toxicity. In this study, the compositions of three interpolyelectrolyte polyplex formulations were quantified by a facile NMR method. Using careful integration of a 1D ^{1}H NMR spectrum with a broad spectral width, the quantities of unbound polymer and polyplexes in solution were determined. Linear polyethyleneimine (PEI) mixed with DNA at polymer amine to DNA phosphate molar ratio (N/P ratio) of 5 revealed an effective binding N/P ratio of 3.5 without excess free polymer. This result is in strong agreement with the stoichiometric number of PEI/DNA binding obtained by isothermal titration calorimetry. The noninvasive nature of this method allows broad application to a range of polyelectrolyte coacervates, opening new opportunities for understanding and optimizing polyelectrolyte complex formation and providing quantitation of complex formation in a single measurement.
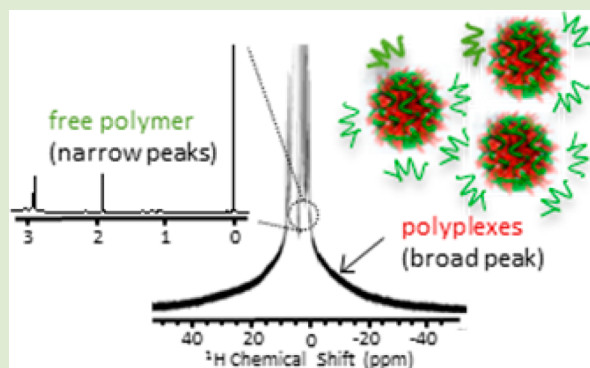
The field of nonviral nucleic acid delivery offers promise for a variety of research and therapeutic applications.[1−3] Over the last two decades a large variety of polymeric delivery systems have been created and studied;[4,5] delivery vehicles are needed to compact nucleic acids into small nanoparticles to facilitate cellular uptake[6] and protect nucleic acids from enzymatic degradation[7,8] and can be designed to target delivery to various tissue types.[9,10] Such delivery systems are typically designed to be polycationic in nature to bind, via electrostatic interactions, with the negative phosphodiester backbone of the nucleic acid. This process is entropically favored via the release of counterions from both the polycation (delivery vehicle) and polyanion (nucleic acid) backbone.[11,12]

While polymeric delivery systems offer promising properties, low gene-transfer efficiency compared to viral vectors is one of the major limitations toward clinical applications. One of the most common strategies conventionally used to improve delivery (cell transfection) efficiency of nucleic acids in vitro is to optimize the polymer amine to DNA phosphate molar ratio (N/P ratio), which is simply increasing the amount of the polycation in the polyplex formulation to obtain the most efficient delivery.[13,14] For most published polyplex-based systems, a large excess of free polymer remains in solution during transfection, yet the exact amount of free polycation is rarely known due to difficulties in characterization and in isolating polyplexes from the free polymer. Thus, in vitro and in vivo assay results (both delivery efficiency and toxicity) are routinely reported without knowledge of the amount of free polymer in solution. It has been theorized that the free polymer plays a central role in many physiochemical and biological parameters, for example, increasing biological membrane permeability,[15,16] delivery efficiency,[17,18] and toxicity.[19] However, the exact role of free polymer concentration is not known.

Several groups have shown that transfection of polyethyleneimine (PEI)-mediated gene delivery in cells is improved at N/P ratios higher than unity (typically 5−7).[20−22] In addition, DNA delivery studies performed on carbohydrate-based poly(amidogalactaramine) G4 and lanthanide-containing theranostic polymers showed increases in transfection efficiency with N/P ratio.[13,23,24] However, forming PEI polyplexes at elevated N/P ratios means that a considerable excess of polymer exists, and this clearly contributes to the cellular toxicity profile of PEI polyplex formulations.[25,26] Therefore, quantifying the concentration of unbound polycation present in polyplex formulations

carries deep implications for optimal and nontoxic delivery of nucleic acids in vitro and in vivo.

Researchers have attempted to address the role of free polymer in transfection for a PEI/DNA complex using fluorescence spectroscopy[21,27,28] and UV−vis spectroscopy.[29] However, sample labeling with probe molecules can significantly affect the solubility and composition of samples and hence the accuracy of quantitation by these methods. In addition, PEI/DNA polyplexes tend to aggregate,[30] and purification was needed prior to spectroscopic analysis due to precipitation,[21] thus requiring constraints on as-delivered sample properties.

Here we describe a new and straightforward NMR peak integration method to accurately determine the concentration of unbound polymer as well as the ratio of this free polymer and polyplexes in solution. For polymer-based gene delivery systems, obtaining this information is critical to optimizing efficacy as well as to assessing consistency of formulation/ quality control for advancing these systems toward animal and clinical studies. In such systems, the ~60−200 nm polyplexes in dispersion exhibit broad NMR resonances (Figure 1b), while
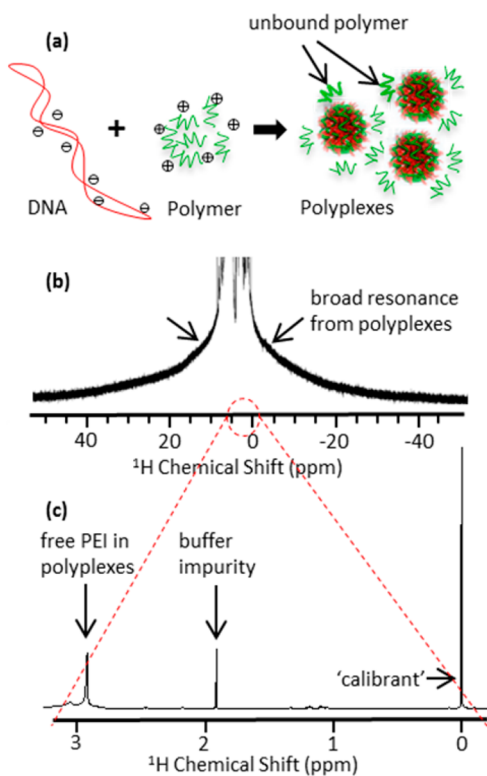

Figure 1. (a) Polyplex formation. (b) ^{1}H NMR spectra of PEI/DNA polyplexes in tris buffer with vertical expansion and wide shift range. The broad resonance ranging from −50 to +50 ppm originates from PEI/DNA complexes. (c) "Conventional" narrow shift range ^{1}H NMR spectrum showing only free PEI in dispersion.

the unbound components show narrow resonances (Figure 1c). The concentration of unbound polymer in solution is obtained by comparing the signal intensity of narrow polymer resonances with that of a dilute internal standard (calibrant). The ratio of free polymer to polyplexes is given by the ratio of the narrow resonance intensity to the broad resonance intensity. The latter has been used to determine, e.g., crystalline/amorphous fraction in semicrystalline polymers.[31]

Using this method, we can also extract the actual binding molar (N/P) ratio of polymer to DNA for any "as-prepared" polyplex formulation. Indeed, this method can be broadly applied to examining coacervates, micelles, and vesicles in solution.

We apply the described method to three different polyplex formulations: (i) 25 kg/mol of linear PEI, (ii) a poly-(glycoamidoamine) (PGAA) polymer delivery vector (G4)[13,18,32] (Figure 2a), and (iii) a nonparamagnetic (lanthanum, La) analogue of a theranostic polymeric vehicle N_4La[23] (Figure 2b), the latter two developed in our group.
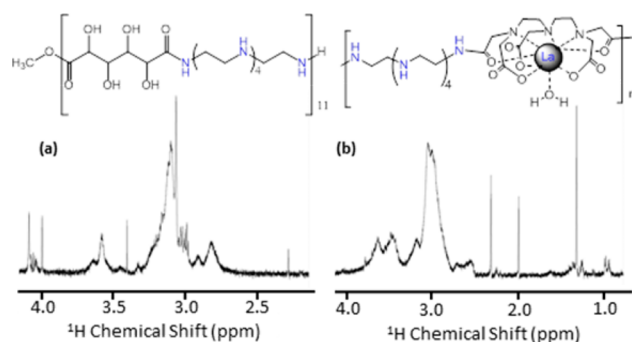

Figure 2. Structures and ^{1}H NMR spectra (narrow shift range) of polyplexes of (a) G4 and (b) N_4La in H_2O.

Prior to NMR experiments, we studied plasmid DNA (pDNA) binding ability to PEI and N_4La using a gel electrophoresis shift assay. Binding and compaction of PEI/ pDNA complexes was evident at N/P ratio higher than 2.5. For N_4La, effective binding took place at N/P ≥ 5 (Figure S7, Supporting Information). Dynamic light scattering (DLS) size measurements of PEI/pDNA polyplexes showed a trend consistent with previous reports on branched PEI polyplexes (Table 1).[27,33]

Table 1. Hydrodynamic Diameter of Polyplexes by DLS Size Measurement[a]

polymer	N/P	D_{hyd}/nm	polymer	N/P	D_{hyd}/nm
PEI	5	210	G4	5	79
	10	120		10	66
	20	77		20	67
pure PEI	∞	7	pure G4	∞	162
N_4La	10	92			
	40	76			
pure N_4La	∞	250			

[a]∞ denotes pure polymer.

For each polymer, polyplex samples were formulated with the same total polymer concentration (170−370 μg/mL), independent of N/P ratio. PEI polyplexes were formulated in tris buffer (10 mM, pH 7), and polyplexes of N_4La and G4 were prepared in nuclease-free water. The addition of 0.009 wt % of sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 to polyplex samples provided the internal standard (calibrant) for NMR measurements. No filtration or other sample pretreatment was necessary prior to NMR quantitation.

We performed all NMR experiments at 310 K using the simple and reproducible presaturation solvent suppression pulse sequence (continuous wave irradiation of 8 mW on water resonance, prior to single pulse acquisition). We experimented

with a variety of more complex water suppression NMR pulse sequences, but the results showed equivalent or larger errors.

By comparing intensities of the narrow resonances from free polymer chain and the calibrant, we quantify the absolute amount of unbound polymer in solution. The intensity of the broad resonance (calculated by subtracting the integrals of free polymer resonances from the total integral from −50 to +50 ppm—see Supporting Information for further description) further yields the concentration of polyplexes in a given dispersion. Any precipitated polymer/DNA in the sample can be thus ignored, which is desirable since that component would not be delivered during treatment. We quantitatively characterized PEI, N_4La, and G4 polyplexes at commonly used N/P ratios for transfection studies using ^1H NMR, despite significant aggregation at lower N/P ratios. No broad resonance appears in the spectrum of the pure PEI polymer sample, which indicates the complete absence of aggregated components.

PEI/DNA polyplexes prepared at N/P = 5 formed significant aggregates (cloudiness in solution) and caused precipitation in buffer. At this N/P ratio, about 70% of polyplexes precipitated to the bottom of the NMR tube. This number is determined by comparing the polymer amount initially added to the sample with the total quantity detected through NMR (from both sharp and broad resonances). The N/P ratio of polyplexes in dispersion without considering free PEI (actual N/P) is 3.5, by comparing the signal intensities of the sharp and broad resonances.

To support the accuracy of this NMR experiment, isothermal titration calorimetry (ITC) measurements were also performed to extract the stoichiometric number of binding between PEI and DNA under the same experimental conditions. Three titrations yielded an averaged stoichiometry of binding, n_{bind}, of 3.5 ± 0.3 (Table 2). This is in excellent agreement with the NMR quantification results above, which show an effective binding N/P ratio = 3.5 for the PEI polyplex sample prepared at N/P = 5.

Table 2. Thermodynamic Parameters for PEI−pDNA Binding from Isothermal Titration Calorimetry

	average	std. dev.	% RSD
n_{bind}	3.5	0.3	8.1
K (M^{-1})	4.94×10^5	2.01×10^5	40.6
ΔH (kJ/mol)	2.16	0.56	26.1

Furthermore, we studied higher N/P ratios of 10 and 20 for PEI, as well as a range of N_4La and G4 polyplex formulations. Figure 3 shows a summary of both the unbound polymer percentage and the effective N/P for the polyplexes as a function of polymer type and "as-prepared" N/P. For PEI, 16% and 27% of the originally added polymer remained unbound at N/P 10 and 20. Accordingly, the actual or effective N/P ratios of the polyplexes are 8 and 17 instead of N/P 10 and 20, respectively. Interestingly, this indicates that significant overcharging of these polyelectrolyte complexes does, in fact, occur. Because precipitation was not observed in these two samples, all polymer species were detected by NMR. Specifically, the sum of signal intensities from the sharp and broad resonances should equal the total quantity of originally added polymer. This equality holds true (with <10% discrepancy), further validating the use of this broad resonance for quantitation. Indeed, incorporating broad resonances into integration is essential for quantifying compositions of samples exhibiting
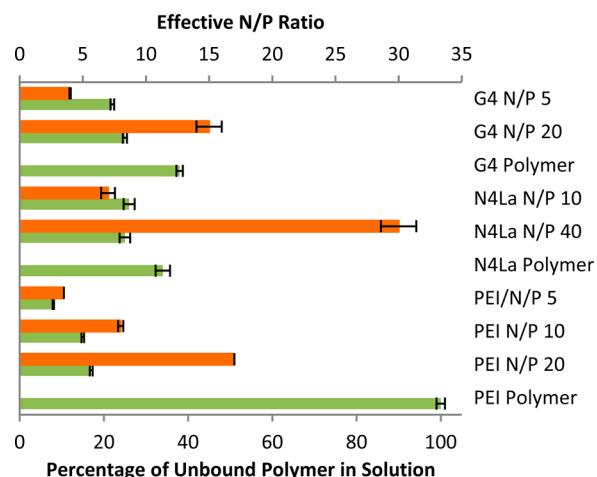

Figure 3. Percentage of unbound polymer in polymer and polyplex solutions (green) and effective N/P ratios of polyplexes (orange), both as a function of as-prepared N/P.

precipitation. Otherwise, polyplexes in dispersion cannot be quantified separately from those precipitated without isolating each polymer species.

To extend these studies to other polymeric delivery systems, we also investigated G4 and N_4La polymers. The repeat unit of G4 polymer consists of a galactose sugar and a PEI-like oligoethylene amine group containing four secondary amines along the backbone (Figure 2a). G4 has gene delivery efficiency comparable to linear PEI but with lower toxicity.[18] DLS shows (Figure S4, Supporting Information) that the G4 polymer alone (no pDNA) forms aggregates of 100−200 nm in diameter in dispersion in its pure water solution; however, the NMR measurements show that only 60% of the polymer exists in these aggregates, and the rest is free unimers. In contrast, for G4/pDNA polyplex solutions, diameters <100 nm (Table 1) were found, indicating that significant compaction of both the pDNA and the polymer aggregates occurs during polyelectrolyte complexation. The complete binding and compaction of DNA by G4 occurs at N/P 4 for the sample prepared at N/P 5 (18% unbound polymer).

The N_4La polymer is a nonparamagnetic analogue of a previously published paramagnetic gadolinium chelate used for direct tracking of gene delivery via magnetic resonance imaging (Figure 2b).[23] DLS measurements of N_4La polymer in pure water show the presence of free polymer (<10 nm) and polymer aggregates (200−300 nm) in solution (Figure S6, Supporting Information). NMR measurements show that 65% of N_4La polymer forms aggregates. The concentrations of free polymer in N_4La polyplex solutions prepared at N/P 10 and N/P 40 are similar (19% and 18%), which indicates enhanced binding between N_4La and DNA with excess polymer added at N/P 40. DLS measurements showed smaller particle size of N_4La polyplex aggregates at N/P 40 compared to N/P 10 (Table 1), which also indicates stronger binding of the N_4La polymer to DNA at N/P 40.

This ^1H NMR quantitation method provides a systematic approach to determine the amount of all polymer species in polyelectrolyte coacervates, in particular, polymer/nucleic acid solutions. This method offers a significantly simplified quantitative analysis of unbound and complexed polymers in polyplex solutions in one measurement, and without the need for disturbing the sample solution. Consequently, it gives better

accuracy and more consistent test conditions compared to other spectroscopic methods for systematic investigations of polymer/nucleic acid systems. Relatively short experimental time of NMR measurements (less than 2 h) is also beneficial for degradable samples. The minimum free polymer concentration detected by NMR in this study is of the order of 10 μg/mL, and this would be improved significantly through the use of more sensitive NMR instruments (e.g., cryoprobe, higher field).

We have demonstrated the utility of a new protocol for sensitive quantification of free polymer in solutions of polycation/DNA gene delivery vectors and theranostic agents. This method will also allow for characterization of compositions for other vesicles and micelles. For polymer-based gene delivery systems, such a simple quantitative analysis will offer critical information to optimize drug delivery efficacy as well as to assess regulatory compliance and quality control.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information
This includes details of synthesis of the N_4La polymer, SEC, and gel electrophoresis characterization of N_4La and PEI, ITC experiments, and NMR integration and signal components. 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.

■ ACKNOWLEDGMENTS

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