

## Sensitive NMR Detection of Cationic-Polymer-Based Gene Delivery Systems Using Saturation Transfer via Proton Exchange

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Received March 19, 2001

Revised Manuscript Received July 24, 2001

Cationic polymers (CPs) have become increasingly important as nonviral DNA delivery systems for potential use in gene therapy.<sup>1</sup> As such, it would be useful if low concentrations of these compounds could be detected with sufficient sensitivity to allow noninvasive visualization of gene delivery or antibody targeting in vivo. To accomplish this using MRI, it has been necessary to label these compounds with (super)paramagnetic tags.<sup>2–4</sup> However, the addition of metallic labels is often cumbersome and may increase the overall toxicity of the delivery systems. Exploiting the inherent properties of amide protons that rapidly exchange with water, we show that enhancements in sensitivity by factors as large as 500,000 are possible, allowing the detection of micromolar concentrations of macromolecules with the molar sensitivity of water. This result suggests the possibility of a new family of nonparamagnetic macromolecular MRI contrast agents with contrast based on the presence of multiple water-accessible amide groups.

Over the course of the past decade, Balaban and co-workers<sup>5</sup> have investigated exchange-based saturation-transfer effects and, by studying the reduction of the water signal, have been able to

indirectly detect 50–100 mM concentrations of small molecules. However, such detection sensitivities are still several orders of magnitude below those achievable with contrast agents such as superparamagnetic tags<sup>2–4</sup> and laser-polarized noble gases.<sup>6</sup> The latter have shown the largest sensitivity enhancements ever reported for NMR, namely close to 5 orders of magnitude. We here show that even larger gains are attainable for the CPs, which contain multiple protons of similar resonance frequency (chemical shift). Because such protons can be simultaneously saturated, their total effective molarity is much higher than that of the molecule itself, allowing for the polymer to act as a “saturation amplifier”. For fast exchange with respect to NH longitudinal relaxation ( $k \gg 1/T_{1\text{NH}}$ ), it can be derived that the proton-transfer enhancement (PTE) is:

$$\text{PTE} = \sum_i \frac{\alpha_i k_i N_i M_w}{(1 - x_{\text{CP}})R_{1\text{wat}} + x_{\text{CP}}k_i} (1 - e^{-[(1 - x_{\text{CP}})R_{1\text{wat}} + x_{\text{CP}}k_i]t_{\text{sat}}}) \quad (1)$$

in which  $\alpha$  is the saturation efficiency ( $0 < \alpha < 1$ ),  $k$  the pseudo-first-order forward rate constant,  $N$  the number of exchangeable protons of a particular type per molecular weight unit (see Table 1),  $M_w$  the molecular weight, and  $x_{\text{CP}}$  the fractional concentration of exchangeable protons for the CP. The exponential term describes the influence of back-exchange and the longitudinal relaxation rate ( $R_{1\text{wat}} = 1/T_{1\text{wat}}$ ) of water protons on the buildup of this effect during the length of the saturation period ( $t_{\text{sat}}$ ). The summation index  $i$  is over different types of macromolecular NH protons, for example, amide, primary, and secondary amines of equal chemical shift, for which the exchange rates can differ. For instance, different generations of Starburst PAMAM dendrimers (SPD- $g$ ) have one  $\text{NH}_2$  group per surface group (Chart 1) and  $2s - 4$  extra amide protons in the individual branches, in which  $s$  is the number of surface groups ( $s = 2^{g+2}$ ;  $g$  = generation number). The total number of exchangeable protons is thus  $4s - 4$ , corresponding to 508 protons for a generation-5 dendrimer (SPD-5; Table 1).

To test the above principles, we determined PTE for several CPs (Table 1). Samples were prepared in aqueous solution (95% 0.01 M phosphate buffered saline (PBS), 5%  $\text{D}_2\text{O}$  by volume) at concentrations set to keep  $x_{\text{CP}}$  of detectable exchangeable protons similar between samples. To visualize the saturation transfer effect of the exchangeable protons, we acquired so-called  $z$ -spectra,<sup>7</sup> or CEST-spectra,<sup>5c</sup> in which the reduction in the water signal due to saturation transfer is measured as a function of NMR frequency offset. In  $z$ -spectra, the reference frequency for water is set at 0 ppm, which corresponds to direct saturation of water. If at any frequency there are exchangeable protons at appropriate concentration and exchange rate, the effect becomes visible through attenuation of the water line. The resulting  $z$ -spectra in Figure 1 show no noticeable saturation transfer effect for PAA and PEI, while effects of different magnitude are measured for PLL, PLE, and SPD-5. On the basis of the data in Table 1, this result indicates that only the amide protons are in the appropriate  $pK_a$  range ( $pK_a \approx 4$ ) to be visible in the NMR spectrum as a separate resonance. This feature of exchanging sufficiently slow on the NMR time scale is a principal requirement for the present approach. When exchange is too fast, a single resonance that is fractionally weighted between exchange sites will be found, coinciding with

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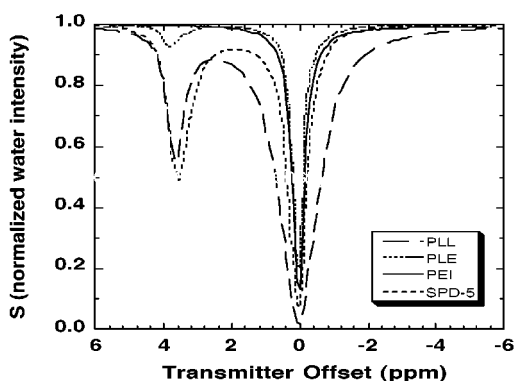
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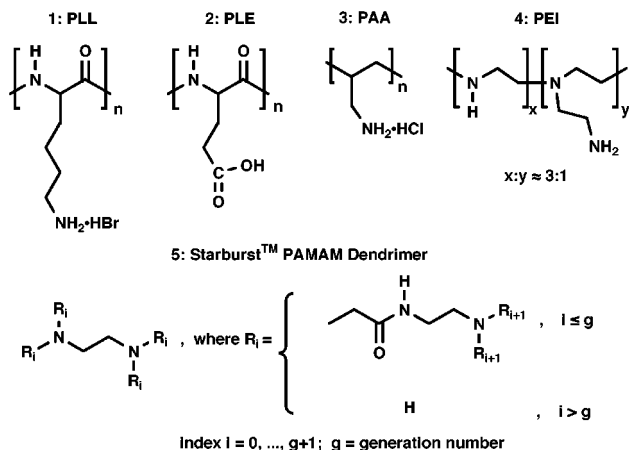
**Table 1.** Cationic Polymer Data and Results for Saturation Transfer and Exchange Properties (pH 7.3–7.4,  $T = 37\text{ }^{\circ}\text{C}$ )<sup>a</sup>

	$M_w$ kD	concentration $\mu\text{M}$	$N$ (amide) protons/kD	$N$ (NH) <sup>b</sup> protons/kD	$N$ (NH <sub>2</sub> ) protons/kD	$k^d$ s <sup>-1</sup>	$x_{\text{CP}} \times 10^3$	PTE	$(S_0 - S_{\text{sat}})/S_0$	
									obsd	calcd <sup>f</sup>
PLL	488	100	4.78	0	9.57 <sup>c</sup>	140	2.11	586,431	0.43	0.53
PLE	70	500	6.62	0	0	10	2.10	15,568	0.07	0.07
PAA	70	300	0	0	21.61 <sup>c</sup>	<i>c</i>	N/A	<i>c</i>	0	0
PEI	750	150	0	4.64 <sup>c</sup>	9.29 <sup>c</sup>	<i>c</i>	N/A	<i>c</i>	0	0
SPD-5	28.825	1000	8.74	0	8.88 <sup>c</sup>	77 <sup>e</sup>	2.29	44,080	0.51	0.40

<sup>a</sup> Abbreviations: poly-L-lysine (PLL), poly-L-glutamate (PLE), polyallylamine (PAA), polyethylenimine (PEI), Starburst PAMAM dendrimers (SPD-5). <sup>b</sup> Nonamide NH protons <sup>c</sup> Exchangeable protons not detectable in spectrum <sup>d</sup> Measured with the WEX-filter<sup>8b</sup> approach. <sup>e</sup> Wide resonance containing multiple amide protons with different exchange rates. <sup>f</sup> Using eq 2,  $\alpha = 1$ , and  $T_{1\text{wat}} = 3.86$  s (determined using an inversion recovery experiment).



**Figure 1.** Water attenuation due to selective radio frequency saturation as a function of chemical shift with respect to water, which is set at 0 ppm ( $z$ -spectrum). The curves for PAA and PEI coincide and only the one for PEI is displayed. The pulse sequence consisted of a continuous low-power rf saturation (500 MHz VARIAN spectrometer;  $t_{\text{sat}} = 10$  s; power 100 Hz; interscan delay 17 s).

**Chart 1.** Structural Formulas

water, and no targeted detection is possible. Also, exchange should be slow enough to allow sufficient saturation of NH protons before exchange. NMR visibility for the CP protons was checked using a flip-back approach<sup>8</sup> to acquire spectra in which exchangeable protons are not suppressed. The results (data not shown) indeed give only measurable exchangeable protons in the spectra for PLL, PLE, and SPD-5. When integrating the peak areas and using the aliphatic protons as intensity reference, the intensity of the exchangeable protons agrees with that expected for the amide groups. This  $pK_a$  limitation needs to be taken into account when designing proton-exchange-based contrast agents.

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Saturation effects were measured independently of the shape of the water line by taking the ratio of the water signal intensity with ( $S_{\text{sat}}$ ) and without ( $S_0$ ) saturation of the exchangeable groups, using the opposite side of the water line as reference intensity.<sup>5</sup> The resulting ratio should be related to the PTE via:

$$(1 - S_{\text{sat}}/S_0) = \frac{[\text{contrast agent}] \cdot \text{PTE}}{2 \cdot [\text{H}_2\text{O}]} \quad (2)$$

The data in Table 1 indeed show good agreement between calculated and observed effects. The reason that the water intensity reductions for SPD and PLL are comparable, despite the fact that the exchange rate for PLL ( $140\text{ s}^{-1}$ ) is much larger, is that back-exchange from saturated water protons to the PLL is significant. The fact that the signal reduction is still overestimated by about 20% may be due to exchange being too fast to allow full saturation before exchange, thereby reducing  $\alpha$  (assumed to be 1). The underestimation of the SPD signal reduction is attributed to the fact that the actual exchange rate may be larger than the measured value. NMR spectra acquired at lower pH show that there are three different amide groups that partially overlap in chemical shift in the NMR spectrum, each of which has a different exchange rate that contributes. At physiological pH, however, it was difficult to resolve the broad signals and to determine the individual exchange rates.

In vivo, the asymmetry of the  $z$ -spectrum for exchangeable protons will be essential to separate the CP effect from the magnetization transfer contrast (MTC)  $z$ -spectrum, which is approximately symmetric. MTC and direct water saturation are separate from but additional to the exchange effect, and saturation power should be optimized to minimize these effects with respect to exchange transfer. We expect that this will be accomplished with saturation powers that are less than for MTC. High magnetic fields are beneficial for this new contrast mechanism, because the amide protons are better resolved and  $T_{1\text{wat}}$  is longer than at low field. For instance,  $T_{1\text{wat}}$  in vivo is about 1 s at 1.5 T, leading to effects that are about 30–40% of the effects measured here at 11.7 T.

In summary, we have shown that micromolar concentrations of CPs can be detected exploiting the molar sensitivity of water. These results should stimulate the tailored design of a family of polyamide-based contrast agents that are optimized with respect to the number of selectively saturable exchangeable protons per molecular weight unit. We foresee that agents with such a maximum number of exchangeable protons in the correct  $pK_a$  range should provide an additional order of magnitude of enhancement. We expect these compounds to become suitable as markers for the monitoring of gene delivery, for cellular labeling, and as pH probes.<sup>5c,8a</sup>

**Acknowledgment.** This research was supported, in part, by NIH grants RR11115 and NS31490. We are grateful to Drs. James Pekar and Xavier Golay (F.M. Kirby Research Center) for helpful discussions.

JA0158455