^{13}C Saturation Transfer Difference (STD)-NMR Experiments Using INEPT Polarization Transfer

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Abstract

Saturation-Transfer Difference (STD)-NMR spectroscopy has been widely used to screen potential ligands for binding to large receptor molecules. The STD-NMR experiment is typically based on a proton NMR spectrum, which can suffer from spectral overlap, leading to missing information in STD-based epitope mapping. Two-dimensional STD-NMR experiments can alleviate spectral overlap, but are time consuming. Here we examine the feasibility of saturating protons in a receptor molecule and observing the STD effect on nearby carbon nuclei after transferring polarization from protons to carbons using the Insensitive Nuclei Enhanced by Polarization Transfer (INEPT) pulse sequence. We show that under favorable conditions, a ^{1}H->^{13}C STD-INEPT experiment can give information similar to that obtained from a two-dimensional heteronuclear experiment, but in significantly less time. The STD-INEPT experiment could be especially useful when studying mixtures of ligands in which the peak positions in the proton and HSQC spectrum change significantly, and in particular when using high-throughput, automated methods to analyze the data.

Introduction

The Saturation-Transfer Difference (STD)-NMR experiment has been widely used in the drug discovery process to screen potential drug candidates against a target protein receptor [1]. This experiment [2,3] consists of collecting two NMR spectra: an on- and off-resonance spectrum. In the on-resonance spectrum, the sample is saturated with radio frequency irradiation at a frequency at which the receptor is expected to resonate. This saturation is applied for a set amount of time (usually several seconds) before beginning the standard NMR experiment. During the saturation time, the receptor protons become saturated, and some of this saturation is transferred to protons on ligands that interact with the receptor during the saturation time. This leads to a decrease in the peak intensity for those ligands that interact with the receptor. In the off-resonance spectrum, irradiation is performed for the same amount of time at a frequency that is far from the resonances of either the receptor or ligand. The off-resonance spectrum is a reference spectrum, and ligands that do not interact with the receptor during the saturation time are expected to have the same peak intensity in the on- and off-resonance spectra. Subtracting the on-resonance spectrum from the off-resonance spectrum, therefore, results in an STD difference spectrum containing NMR peaks from only those ligands that interact with the receptor. For ligands that do interact with the receptor, the STD effect of each NMR peak is defined as the intensity of the peak in the difference spectrum divided by the intensity of the peak in the reference spectrum [3]. An STD buildup curve can be constructed by plotting the STD effect as a function of saturation time. The STD effect is expected to increase with increasing saturation time before reaching a maximum STD effect. Angulo *et al.* [4] have shown that when calculating binding constants from STD-NMR experiments, the initial slope of the STD buildup curve is a better measure than one STD effect at a single saturation time.

The advantage of the STD-NMR technique is that it can quickly and clearly identify binding ligands in the presence of a large library of binding and non-binding ligands. The STD-NMR technique is simple to implement and is included in most commercial NMR instruments and software. Beyond simple screening experiments, the STD-NMR technique also has the potential to be used for epitope mapping, in other words to determine which part of the ligand molecule is located closer to the receptor site in the bound conformation [3]. We have previously shown that the STD-NMR technique can be used to examine binding between small molecules and nanoparticle receptors [5-7].

The most common STD-NMR experiment is a simple one-dimensional ^{1}H STD experiment. This experiment is straightforward and can quickly provide both screening and epitope mapping information. However, in the case of epitope mapping, spectral overlap in 1D ^{1}H NMR can be a problem, as STD effects cannot be obtained from each individual site in an overlapped peak. Two-dimensional STD-NMR experiments, including STD-TOCSY [8] and STD-HSQC [9-11], are also common, and can be useful for resolving overlapping sites. Two-dimensional NMR techniques are time consuming, though, due to the requirement for collecting multiple one-dimensional experiments to obtain the second dimension. This time requirement may be ameliorated by using non-uniform sampling, but is multiplied when multiple spectra at different saturation times are required to construct an STD buildup curve.

The increased chemical shift dispersion of carbon compared to proton will often resolve sites that are overlapping in the proton spectrum, so one-dimensional ^{13}C STD experiments are attractive. A homonuclear carbon STD experiment, however, would be hindered by the low natural abundance of the ^{13}C nucleus. The low occurrence of ^{13}C isotopes prevents the efficient spin diffusion which is necessary for saturation to spread to the entire receptor and transfer to the ligand.

Instead, we have chosen to saturate receptor protons, followed by polarization transfer to carbon using the Insensitive Nuclei Enhanced by Polarization Transfer (INEPT) pulse sequence.

Using INEPT to transfer polarization from proton to carbon [12] or fluorine [13] in an STD experiment has been previously suggested. Polarization transfer to carbon has advantages over transferring the polarization to fluorine. Observing carbon rather than fluorine removes the need for ^{19}F labeling, and ^{1}H -> ^{13}C INEPT can query all protonated carbons in the ligand, rather than just one or two fluorinated sites. Both INEPT methods have the advantage that water suppression is not necessary [12].

In the current work, we quantify the STD effect from ^{1}H -> ^{13}C INEPT STD NMR (hereafter STD-INEPT) and compare it with results from ^{1}H STD NMR and STD-HSQC NMR experiments for a series of compounds interacting with polystyrene nanoparticles. We examine the feasibility of using STD-INEPT, and under which conditions this experiment may be preferable to the two-dimensional STD-HSQC experiment.

Experimental Methods

Carboxylate-modified (CML) polystyrene latex beads (4 % w/v, 0.020 μm) and deuterium oxide (Acros Organics) were purchased from ThermoFisher Scientific (Waltham, MA, USA). D-phenylalanine, pentanol, and L-tryptophan, L-threonine, and L-arginine were purchased from Millipore Sigma (Burlington, MA, USA). All reagents were used as received without further purification.

Samples were prepared by dissolving each analyte in D_2O and then adding the polystyrene bead suspension so that the final concentration was 100 mM phenylalanine, 240 mM pentanol, and 35 mM each of arginine, threonine, and tryptophan. The final weight percent of polystyrene latex beads was 0.4 % (w/v) in the phenylalanine and amino acid samples and 2 % (w/v) in the pentanol sample. Samples were transferred to 5 mm od NMR tubes (Norell, Inc, Morganton, NC, USA) for measurement. Control samples were prepared with the same concentration of analyte but with an equal volume of de-ionized H_2O added in place of the polystyrene beads.

All NMR experiments were performed on a Bruker NEO 500 MHz NMR spectrometer with a Prodigy nitrogen-cooled cryoprobe. Standard Bruker pulse sequences were used for the ^{1}H STD and STD-HSQC experiments. Standard proton and carbon 90° pulse lengths were 12.5 μs and 10 μs, respectively. The ^{1}H->^{13}C INEPT STD experiments were conducted by modifying the standard ^{1}H->^{13}C INEPT sequence to include saturation on the proton channel at a specified frequency prior to the first ^{1}H 90° pulse. On- and off-resonance spectra were collected in a non-interleaved manner for the STD-INEPT experiments. For the STD-HSQC experiments, the STD effect was calculated by comparing the difference intensity to the intensity of the same peak in a standard HSQC experiment, without any saturation. Saturation was achieved using a train of 50-ms Gaussian pulses. On-resonance saturation was done at 12 ppm and off-resonance saturation was done at 200 ppm. Recycle delays were 12 seconds for the phenylalanine sample and 5 seconds for the pentanol and amino acids mixture samples for all experiments.

For the ^{1}H STD-NMR experiments, a spectral width of 11.75 ppm and acquisition time of 3 s were used. 16 scans each with on- and off-resonance saturation were collected in an interleaved manner following 4 dummy scans.

For the STD-HSQC experiment, spectral widths of 15.61 ppm in the proton dimension and 165 ppm in the carbon dimension were used. 8 scans each with on- and off-resonance saturation were collected following 16 dummy scans. 128 increments were collected in the second dimension. The two-dimensional FIDs were zero-filled to 8192 x 8192 and line broadening of 0.3 Hz was applied before Fourier transformation.

For the ^{1}H->^{13}C INEPT STD experiments, a spectral width of 248.37 ppm and acquisition time of 1.04 s were used. For phenylalanine and the amino acid mixture, 512 scans each with on- and off-resonance saturation were collected following 16 dummy scans. For the pentanol sample, only 64 scans each with on- and off-resonance saturation were collected. The FIDs were zero-filled to 131072 and 5 Hz of line broadening was applied prior to Fourier transformation.

Results and Discussion

As a test of the STD-INEPT method, in Figure 1 we compare the STD buildup curves of phenylalanine interacting with carboxylate-modified polystyrene beads using the standard ^{1}H STD NMR experiment, the two-dimensional STD-HSQC experiment, and the STD-INEPT method. As we have seen in our previous work [7], the aromatic protons in phenylalanine have significantly higher STD effects than the α and β protons. This trend is also seen when the STD-HSQC experiment is used to construct the STD buildup curve. With the STD-HSQC experiment, however, the maximum STD effects are all lower, and the STD effects reach their maxima more quickly. The STD buildup curves that were constructed using the STD-INEPT experiment are in good agreement with those that were constructed using the STD-HSQC experiment, albeit with slightly more scatter in the points. For these buildup curves, 512 scans (each for the on- and off-

resonance spectra) were collected for the STD-INEPT experiment for a total acquisition time of 4 hours to obtain each point in the buildup curve. For the STD-HSQC experiment, each point in the buildup curve required 7 hours.

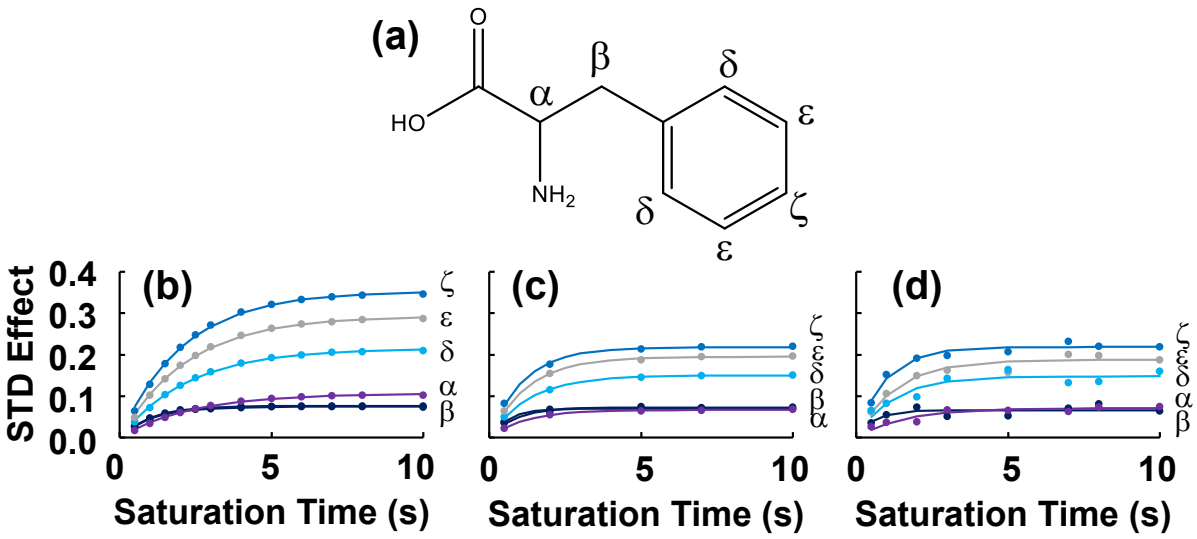

Figure 1. STD buildup curves for phenylalanine interacting with the surface of carboxylate modified polystyrene beads. (a) Chemical structure of phenylalanine with proton peak assignments, (b-d) STD buildup curves constructed from (b) a standard one-dimensional ^{1}H STD-NMR experiment, (c) an STD-HSQC experiment, and (d) an STD-INEPT experiment.

Having shown that the STD-INEPT experiment gives the same results as the STD-HSQC experiment for phenylalanine interacting with carboxylate-modified polystyrene beads, we then moved on to a system in which the ^{1}H STD NMR experiment does not provide sufficient resolution for our purposes. Previous work [5] has indicated that alcohols such as ethanol and isopropanol interact with the surface of carboxylate-modified polystyrene beads as indicated by observable STD effects. We wanted to use the epitope mapping capabilities of the STD-NMR experiment to determine the orientation of a long-chain alcohol, such as pentanol (Figure 2a), when interacting with the nanoparticle surface. Unfortunately, since the CH_2 groups of a long aliphatic chain are in

similar electronic environments, there is significant overlap in the ^{1}H NMR spectrum. For example, as can be seen in Figure 2(b), the ^{1}H NMR spectrum of pentanol shows only four distinct peaks. Longer n-alcohols, such as octanol, experience even greater spectral overlap in their ^{1}H NMR spectra. In the ^{13}C NMR spectrum of pentanol, shown in Figure 2(c), however, all five carbon sites are resolved. Thus, due to the larger chemical shift dispersion of ^{13}C as compared to ^{1}H, we expect that an STD-INEPT experiment could provide useful additional information in the case where the ^{1}H NMR spectrum experiences significant spectral overlap.

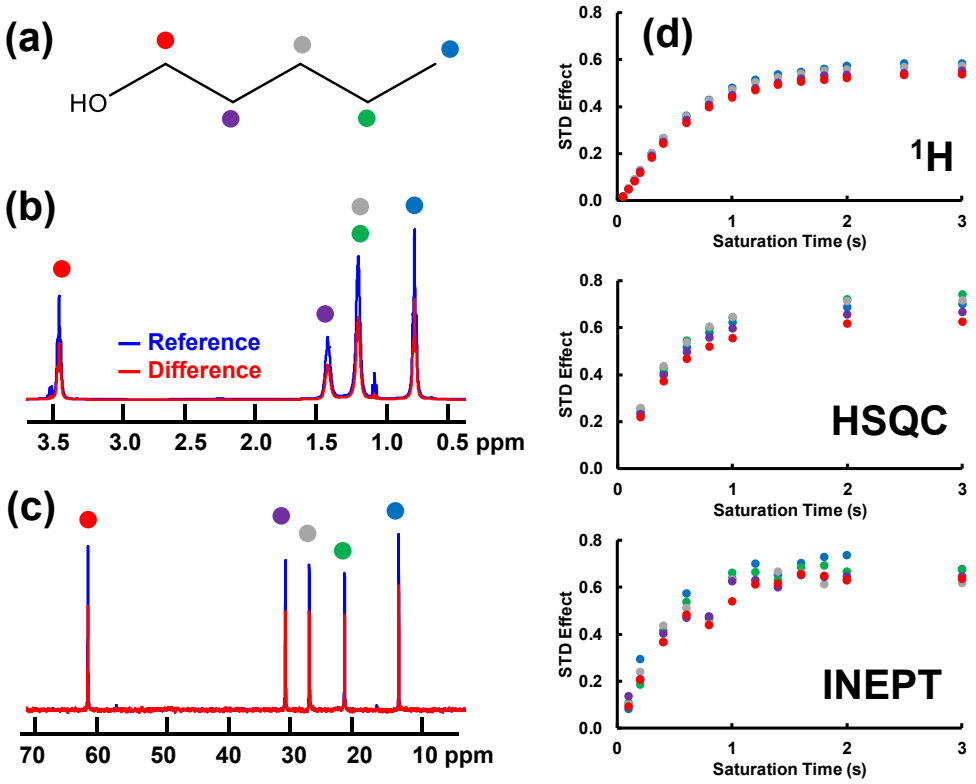

Figure 2. Comparison of various STD-NMR techniques for examining pentanol binding to the surface of carboxylate-modified polystyrene nanoparticles. (a) Chemical structure of pentanol with peak assignments. (b) ^{1}H STD-NMR spectrum of pentanol (c) STD-INEPT spectrum of pentanol. In (b) and (c), blue is the reference spectrum and red is the difference spectrum obtained at a saturation time of 3 seconds. (d) Comparison of STD buildup curves obtained from ^{1}H STD, STD-HSQC, and STD-INEPT experiments. The STD-INEPT buildup curve is in agreement with that from the STD-HSQC experiment, but each STD-HSQC experiment took 3 hours, while each STD-INEPT experiment only took 16 minutes.

In Figure 2(d), we compare the ^{1}H STD-NMR, STD-HSQC NMR, and STD-INEPT buildup curves for pentanol interacting with carboxylate-modified polystyrene beads. The ^{1}H STD-NMR spectrum contains only four well-resolved sites, while the STD-HSQC and STD-INEPT experiment are able to resolve all five CH_n sites. The buildup curve for the STD-INEPT experiment is in good agreement with that for the STD-HSQC experiment, indicating that the STD-INEPT NMR method can give similar results to the standard two-dimensional method. As was the case for phenylalanine above, the buildup curves from the STD-HSQC and STD-INEPT experiments are not in quantitative agreement with the buildup curve from the ^{1}H STD experiment, but the trends are the same. Notably, due to the high signal-to-noise ratio of the pentanol carbon spectrum, only 64 scans were collected for this experiment, for a total experimental time of 16 minutes for each STD-INEPT experiment. The STD-HSQC experiments, on the other hand, each took 3 hours. In the case of pentanol, all CH_n groups have similar STD buildup curves. This indicates that pentanol interacts with the beads in such a way that all CH_n groups are located approximately the same distance from the surface of the bead, presumably by lying flat on the surface of the beads rather than adopting a brush-like conformation in which the pentanol molecule extends radially out from the bead surface.

It is not surprising that the STD-HSQC and STD-INEPT experiments give similar buildup curves, since they essentially report the same kind of information – the saturation that has been transferred from the receptor protons to each ligand CH_n group. This is subtly different from the information that is reported by the ^{1}H STD experiment. While in the ^{1}H STD NMR experiment, we observe one STD effect for each proton, in the STD-HSQC experiment or STD-INEPT experiment, we observe one STD effect for each CH_n group. Thus, for phenylalanine and pentanol in Figures 1 and 2, we observe a slightly different buildup curve for the ^{1}H STD-NMR experiment than we do

for the STD-HSQC and STD-INEPT experiments. In the case of phenylalanine, the buildup curves for the STD-HSQC and STD-INEPT experiments have a shorter buildup time (i.e. reach a maximum faster) but lead to a lower maximum STD effect than the buildup curve from the ^{1}H STD experiment. On the other hand, in the case of pentanol, the buildup curves for the STD-HSQC and STD-INEPT experiments also reach their maxima faster, but lead to a slightly higher maximum STD effect than the buildup curve for the ^{1}H STD NMR experiment. The intensity of the carbon peaks in the STD-INEPT experiment will be influenced by the efficiency of magnetization transfer from ^{1}H to ^{13}C in each case, but since the STD experiment relies on collecting an on-resonance and an off-resonance (reference) spectrum, any differences in the efficiency of magnetization transfer between different CH_n groups will be the same in the reference and on-resonance spectrum, and should therefore cancel out when the STD effect is calculated.

One situation in which the STD-INEPT experiment can be especially useful is when studying a mixture of ligands. In addition to the problem of spectral overlap, proton chemical shifts are especially sensitive to environmental effects such as a change in pH that could be caused by addition of different elements of the mixture, which could lead to additional time spent on peak assignment. This peak shift will also influence peak positions in the HSQC spectrum, and may cause difficulty when automated data processing scripts are used. Carbon chemical shifts, on the other hand, have a greater spectral dispersion and are less sensitive to environmental changes, making peak assignment in the presence of multiple components of a mixture more straightforward.

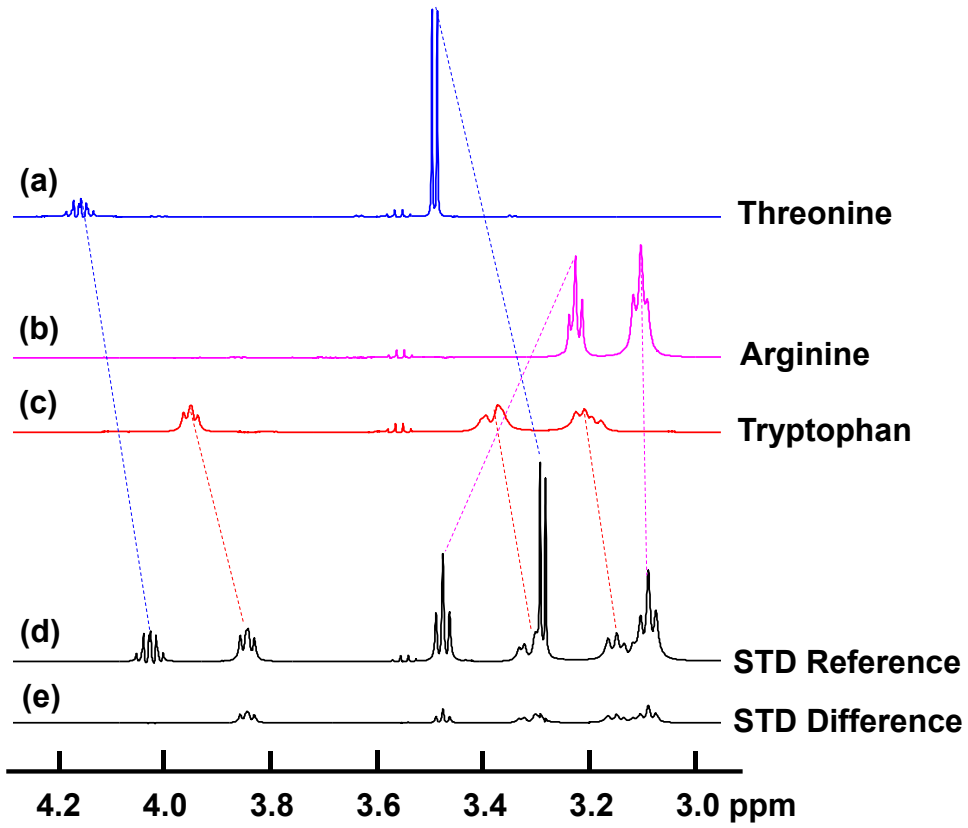

Figure 3. (a-c) A portion of the standard ^{1}H NMR spectra of (a) threonine, (b) arginine, and (c) tryptophan in the presence of 20-nm polystyrene beads. (d) The same region of the ^{1}H STD reference spectrum for a mixture of 35 mM each of tryptophan, arginine, and threonine interacting with 20-nm polystyrene beads. The tryptophan β protons, at 3.2 and 3.4 ppm, overlap with peaks from arginine and threonine, respectively, so that an STD effect could not be calculated for these protons. (e) The same region of the ^{1}H STD difference spectrum showing an STD effect from tryptophan and arginine. An STD effect would be difficult to determine for the overlapping peaks between 3.0 and 3.4 ppm.

A portion of the proton STD-NMR spectrum for a mixture of three amino acids interacting with the surface of carboxylate-modified polystyrene nanoparticle beads is shown in Figure 3. Also shown in Figure 3 are the same regions of the standard proton spectra of the individual amino acids in the presence of the polystyrene beads. As can be seen from comparing the spectra of the individual amino acids with the spectrum of the mixture, there is significant shifting of peak positions in the mixture and significant overlap within this particular spectral region. Obtaining an

STD effect for the tryptophan β protons (at 3.2 and 3.4 ppm), for example, would be difficult due to overlap with threonine and arginine peaks.

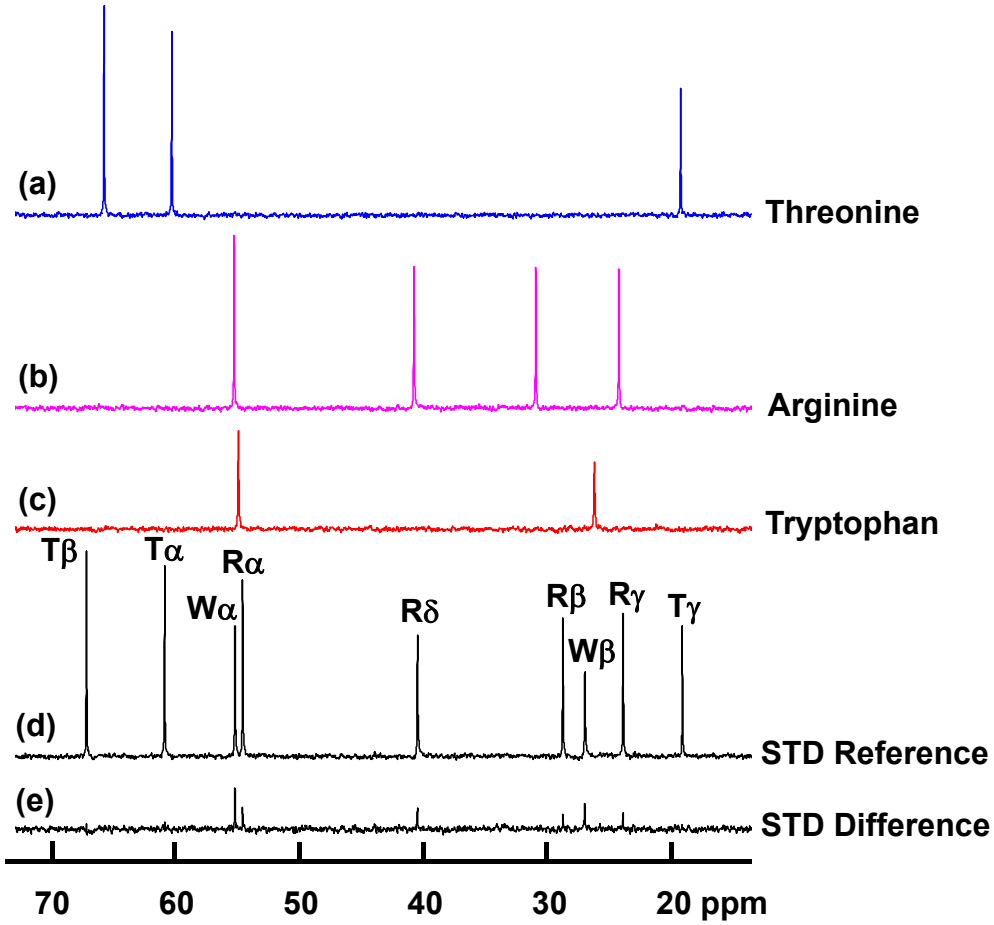

Figure 4. (a-c) A portion of the carbon INEPT spectra of (a) threonine, (b) arginine, and (c) tryptophan in the presence of 20-nm polystyrene beads. (d) The same region of the carbon STD-INEPT reference spectrum for a mixture of 35 mM each of tryptophan, arginine, and threonine interacting with 20-nm polystyrene beads. (e) The same region of the STD-INEPT difference spectrum showing an STD effect from tryptophan and arginine. Carbon peak assignments, using the one-letter code for each amino acid, are listed in (d).

In the STD-INEPT experiment for this mixture (Figure 4), on the other hand, there is much less ambiguity in the peak assignments and no spectral overlap in an equivalent spectral region. One can quickly see that there is no STD effect for threonine, and the STD effects for tryptophan and

arginine can easily be determined. An STD-HSQC experiment for this mixture (not shown) is also able to resolve these sites, but again the STD-INEPT experiment is much faster. Each STD-INEPT experiment for this mixture took 30 minutes while each STD-HSQC experiment took 3 hours. As can be seen from the corresponding STD buildup curves (Figure 5), the STD effects that were determined from the STD-INEPT experiment have larger errors than those determined from the STD-HSQC experiment, but this does not mean that the STD-INEPT experiment is without utility.

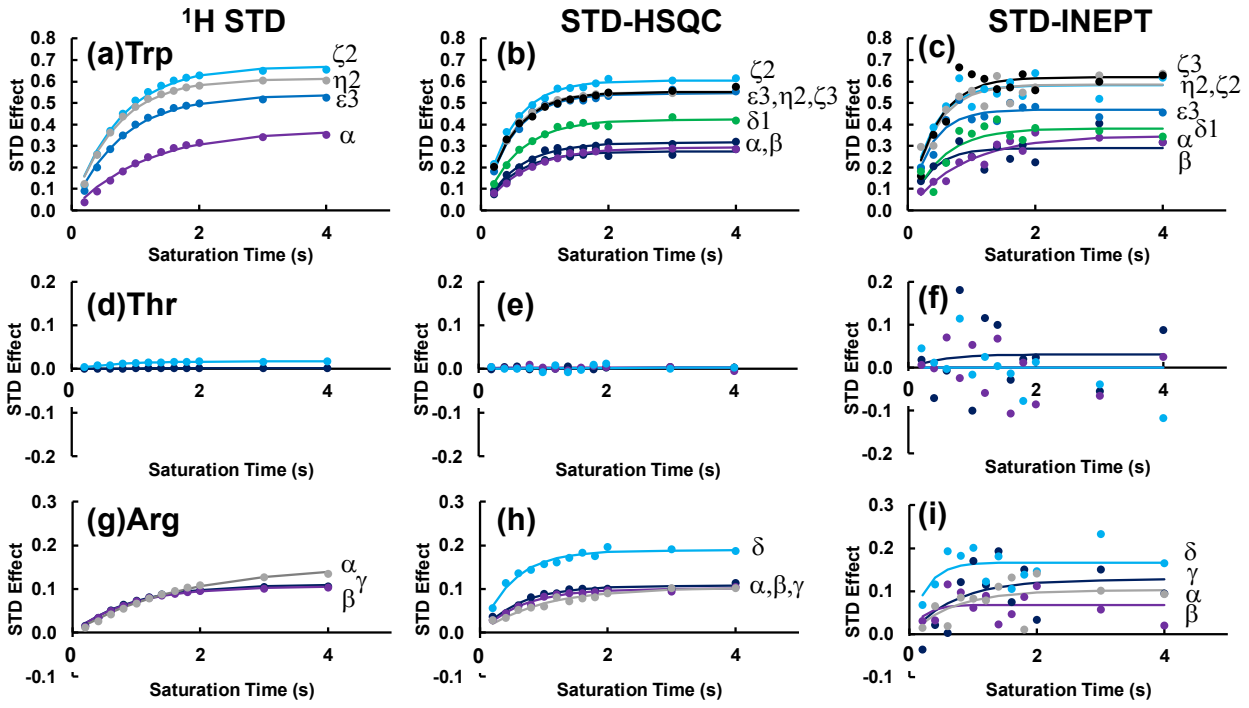

Figure 5. Comparison of STD buildup curves determined from (a,d,g) ^{1}H STD-NMR experiments, (b,e,h) STD-HSQC experiments, and (c,f,i) STD-INEPT experiments for (a,b,c) tryptophan, (d,e,f) threonine, and (g,h,i) arginine in a mixture of the three amino acids interacting with 20-nm polystyrene nanoparticle beads.

The STD-INEPT experiment could be useful in a workflow consisting of using STD-INEPT experiments for initial screening and for preliminary epitope mapping of the most prominent peaks, especially in cases in which the ^{1}H NMR spectrum suffers significant spectral overlap

and/or difficulty in peak assignment. The STD-INEPT experiment would also be beneficial when data analysis requires a one-dimensional spectrum, or when automated data analysis scripts are used. Hyperpolarization combined with STD-INEPT experiments [14] may also be promising in the future to increase signal-to-noise for this experiment, making the errors in the buildup curve determined from the STD-INEPT experiment comparable to those in the buildup curve determined from the STD-HSQC experiment.

Conclusions

In conclusion, we have explored the use of ^{1}H->^{13}C INEPT STD NMR to examine binding between small molecules and polystyrene nanoparticles. We have shown that the STD-INEPT experiment gives STD buildup curves that are qualitatively similar to ^{1}H STD buildup curves, and quantitatively similar to buildup curves based on STD-HSQC experiments. The difference between ^{1}H STD and STD-INEPT buildup curves could be attributed to the subtly different information contained in each experiment, namely the difference between observing the saturation that has been transferred to each proton versus observing the saturation that has been transferred to each CH_n group. Both the STD-INEPT and STD-HSQC experiments can resolve overlapping peaks in proton STD spectra, and don't require water suppression. Under favorable conditions of high signal-to-noise in the carbon spectrum, the STD-INEPT experiment can give the same information as an STD-HSQC experiment in less time. This could be useful for rapid screening and epitope mapping when there is spectral overlap in the proton NMR spectrum, and should be particularly useful when performing an epitope map of a mixture of several compounds.

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Author Contributions

All authors designed experiments, HX and DL performed experiments, all authors analyzed data, LBC wrote the manuscript, all authors commented on the manuscript and approved the final version of the manuscript.

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