SCIFED Publishers

Olga Vinogradova, SF Pharma J, 2018, 1:2

SciFed Journal of Pharmaceutics Journal

Research Paper Open Access

NMR as a Semi-Quantitative Tool for Evaluating Protein Surface Hydrophobicity

Khiem Q Nguyen, Elizabeth Zecca,*Olga Vinogradova, Devendra S. Kalonia

Department of Pharmaceutical Sciences, School of Pharmacy, University of Connecticut, 69 North Eagleville Rd, Storrs, Connecticut 06269-3092, United States

Abstract

Nuclear Magnetic Resonance spectroscopy was applied for evaluation of the surface hydrophobicity of three proteins, Bovine Serum Albumin, α-chymotrypsinogen A and β- lactoglobulin A. The protein surface hydrophobicity was correlated with the binding of small molecular probes selected based upon their aliphatic or aromatic moieties. The interactions were quantified through the transverse relaxation time, T2, where a significant decrease in the transverse relaxation time of the small probe indicated a more pronounced hydrophobic binding to the protein. For all proteins, phenol, an aromatic alcohol, acted as the most informative probe and showed that BSA is the most hydrophobic of proteins studied. The comparison between A-ChtA and B-LgA came inconclusive due the severe instability of A-ChtA in solution. In addition, more common classical approaches for surface hydrophobicity evaluation, HIC and fluorescence spectroscopy, were in agreement with the NMR results. For HIC experiments in particular, two columns were used to further assess the difference in aromatic and aliphatic interactions, confirming the notion of prevalent aromatic binding for all three proteins.

Keywords

Hydrophobicity; NMR Spectroscopy; Proteins; BSA

Abbreviations

A-ChytA - α-Chymotrypsinogen;
ANS - 8-Anilinonaphthalene-1-Sulfonic Acid;
Bis-ANS - 4, 4'-Bis(1-Anilinonaphthalene 8-Sulfonate);
B-LgA - β-Lactoglobulin A;
BSA - Bovineserum Albumin;
CPMG - Carr-Purcell-Meiboom-Gill;
HIC - Hydrophobic Interaction Chromatography;
NMR - Nuclear Magnetic Resonance Spectroscopy;
Prodan - N, N-Dimethyl-6- Propionyl-2-Naphthylamine

1. Introduction

Hydrophobic interactions define key protein properties, including stability, structure, and functionality [1, 2]. Therefore, characterizing the hydrophobicity of a protein would be essential for understanding its behavior. There are two common ways to characterize hydrophobicity in a protein molecule. The net hydrophobicity accounts for all nonpolar side chains present, whereas the surface hydrophobicity deals only with solvent exposed nonpolar side chains [3, 4]. The surface hydrophobicity is a major contributor to physical instabilities, such as protein self-association, aggregation and adsorption to interfaces [5, 6, 7]. The focus of this investigation is to quantitate this property (entropic/dispersion forces etc.) of the protein while eliminating any electrostatic or steric influences.

***Corresponding author:** Olga Vinogradova, Department of Pharmaceutical Sciences, School of Pharmacy, University of Connecticut, 69 North Eagleville Rd, Storrs, Connecticut 06269-3092, United States. E-mail: olga.vinogradova@uconn.edu

Received March 16, 2018; **Accepted** June 21, 2018; **Published** July 05, 2018

Citation: Olga Vinogradova (2018) NMR as a Semi-Quantitative Tool for Evaluating Protein Surface Hydrophobicity. SF Pharma J 1:2.

Different experimental techniques have been used for measuring surface hydrophobicity. Hydrophobic Interaction Chromatography (HIC) is a purification method which provides high resolution and relatively mild solution conditions when compared to other chromatographic methods including Reverse Phase Chromatography [8, 9, 10] Therefore, HIC has been used to compare the surface hydrophobicity of different proteins. In HIC, proteins bind to a stationary phase (column) composed of a nonpolar hydrocarbon chain or phenyl residue. The mobile phase contains a high concentration of salt, such as ammonium sulfate, in order to promote binding between the protein and the weakly nonpolar stationary phase. As the salt concentration decreases, the proteins will elute off the stationary phase following the order of their hydrophobicity, where the least hydrophobic proteins elute first. While HIC can illustrate that one protein is more hydrophobic than another, based on different retention times, it still suffers a number of limitations. First, various experimental conditions, such as the type of column, temperature, salt, and ionic strength of the mobile phase, can considerably increase or decrease retention times of proteins, contributing to the difficulty in interpretation and problems with reproducibility of results [11-16]. As a result, comparing protein hydrophobicity necessitates matching experimental conditions. In addition, proteins in this method are subjected to a very high ionic strength salt buffer, which may screen any potential electrostatic interactions and heighten hydrophobic interactions. As both electrostatic and hydrophobic interactions impact the protein stability, the outcome of the comparison could be misleading: if the charges are masked, hydrophobic interactions may become dominant even if under other circumstances they are not. Furthermore, the size of the protein and the degree of hydrophobic heterogeneity will impact the interactions between the column and the protein. This may lead to a deceiving representation of the hydrophobic characteristics of the molecule as a whole. These caveats are important to keep in mind considering that solution conditions where the proteins will be formulated and stored are not equivalent to HIC conditions.

The use of extrinsic fluorescence probes, such as ANS, Bis ANS, and Prodan, is another method to assess the hydrophobicity of proteins. These probes have a low quantum yield in an aqueous environment, but once the probe enters a hydrophobic environment the quantum yield increases and there is a shift in the wavelength of maximum fluorescence [17]. Although this technique is fast, simple, and nondestructive, it is not a truly reliable measure of surface hydrophobicity. This is due to the fact that the mode of binding of the extrinsic dye to the protein can be different between dyes, which can ultimately affect the hydrophobicity value obtained [18]. Purely aromatic probes may interact through π-π interactions with tryptophan or tyrosine residues, whereas those that are purely aliphatic or those comprising of both aliphatic and aromatic components may bind differently to the protein surface. Along with being structurally distinct, certain probes may also acquire charges in an aqueous solution (i.e. ANS), depending on the pH and ionic strength of the solution. This leads to a potential over/under estimation of hydrophobicity as electrostatic forces can both enhance and reduce the interactions between a dye and a protein [8, 19]. The size of the probes can also limit their ability to detect the surface hydrophobicity. If hydrophobic amino acids are only partially exposed or lay within a narrow pocket, only a smaller nonpolar probe, the one that isn't affected by solution conditions and is small enough to access these residues, would be efficient in exploring this hydrophobic patch.

To conclude, both traditional techniques, fluorescence spectroscopy and hydrophobic interaction chromatography, have a number of experimental problems limiting their ability to provide an accurate value of surface hydrophobicity. The main issue that needs to be addressed is that the values obtained by these methods are only relative numbers, having no real significance to the absolute hydrophobic character of a protein, and have to be compared to the values measured for another protein to be of any use. This stresses the importance of establishing a multi-method quantitative measurement protocol to define real surface hydrophobicity, which provides sufficient sensitivity to differentiate hydrophobic interactions from pure aromatic contributions, independent of experimental conditions.

This paper focuses on application of Nuclear Magnetic Resonance (NMR) to understand the surface hydrophobicity of proteins. NMR is a sensitive and robust technique that can be used to study the binding between a small molecule

(probe) and a larger macromolecule (protein) by observing the transverse relaxation time (T2) [20]. Monitoring the relaxation time of the small molecule in the absence and presence of a protein will reflect the degree of interaction, providing useful information about the hydrophobic surface of the protein. Aromatic or aliphatic small molecules can be used to analyze hydrophobic interactions with the potential to separate the $\pi-\pi$ effect, the ability that was not previously achieved.

In this study, the sensitivity of NMR to measure surface hydrophobicity was explored by comparing the binding between various probes and three well-known proteins. More traditional HIC and fluorescence data for the same three proteins was also obtained and compared with the NMR results. Positive correlation in the relative hydrophobicity measured by different methods was confirmed and substantiated by additional information.

2. Materials and Methods

2.1. Materials

Bovine Serum Albumin (BSA), α-Chymotrypsinogen A from Bovine Pancreas (A-ChytA), and β-Lactoglobulin A (B-LgA) from Bovine Milk, N-Acetyl-L-Leucine Methyl Ester and N-AcetylL-Phenylalanine Ethyl Ester were purchased from Sigma (St. Louis, Mo). Acetyl-Valine-Methyl Ester was purchased from Bachem Americas Inc., N-Acetyl-L-tryptophan Ethyl Ester was purchased from TCI Chemicals and N-Acetyl-L-Tyrosine Ethyl Ester was purchased from MP Biomedicals, LLC. ANS (8-anilino-1-naphthalenesulfonic acid) was purchased from Molecular Probes. The Hiscreen Butyl HP column and Hiscreen Phenyl HP column was purchased from GE Healthcare. Solutions were prepared in a sonicated 15 mM ionic strength (8.5 buffer strength) phosphate buffer (pH 7.0). All buffers and protein stock solutions were filtered through 0.22 μm filters.

2.2. Methods

2.2.1. Sample Preparation

All samples were prepared in the same buffer (8.5 mM sodium phosphate, 15mM ionic strength, pH 7.0, and ~90% D2O) using nitrogen flushed D2O. The probe concentration was held constant at 3 mM, where molar ratios of 1:20, 1:50, 1:100, 1:150, 1:200, 1:400, and 1:1000 correspond to 150 μM, 60 μM, 30 μM, 20 μM, 15 μM, 7.5 μM, and 3 μM of protein (BSA, A-ChytA, or BLgA), respectively. A 1:50 ratio was used (1.5 mM probe and 30 μM protein) for comparative studies between probes. The probes were also investigated alone at the concentrations of 1.5 mM, 3 mM, or 6 mM. None of the samples contained any reference compounds due to initial observations that internal references can also bind to the protein targets, which skew the measurements, while external references significantly complicated the acquisition process due to the problems associated with shimming of two different compartments simultaneously. The probes selected to be tested belong to two major subclasses of compounds: aliphatics and aromatics. The aliphatics consisted of *tert*-butyl alcohol, 1-propanol, and 1-butanol whereas only phenol was used as an aromatic. In addition, capped amino acids were selected to mimic proteinprotein interactions. Similarly, the capped amino acids were chosen based on their aliphatic and aromatic side chains, with the aliphatics being N-acetyl-L-leucine methyl ester, and N-acetyl-Lvaline methyl ester, and the aromatics were N-acetyl-L-phenylalanine ethyl ester, N-acetyl-Ltryptophan ethyl ester, and N-acetyl-L-tyrosine ethyl ester.

2.2.2. Measuring Transverse Relaxation Time

The binding of the probe to the protein was determined by measuring the transverse relaxation time (T2) of the probe. Samples were prepared in 535-PP-7 NMR tubes purchased from Wilmad Labglass (Vineland, NJ) and experiments were performed at 25°C. The water signal was suppressed by presaturation at power level of 6 dB for 3 seconds. The T2 was acquired from array experiments performed by using the Carr-Purcell-Meiboom-Gill (CPMG) T2 pulse sequence without temperature compensation [21]. The acquisition delay was set to 25 seconds, no sample heating was observed under these conditions as judged by the lack of temperature-dependent perturbations in chemical shifts. No alterations in peaks shapes due to Jcoupling [22] was noticed either. The bigtau parameter, which is the time between the initial pulse and data acquisition, was non-uniformly distributed with larger bias to the smaller time values to improve exponential fit. The bigtau sets were not

identical between experiments due to the variability of the relaxation of the probes (see the supporting information for bigtausets).

The integral for the probe peak of interest was taken in each spectrum of the array. Errors originating from overlapping peaks were minimized by base-line correction, which involves subtracting a spectrum of the protein alone from the spectrum of the probe with additional correction factors to account for concentration differences. The intensities for the peak of interest were also taken and used to compare exponential fitting parameters (see supporting information). The fit was carried out using the VnmrJ v3.2 T2 analysis module. The equation used for the fit was

$$y = m_0 * e\left(\frac{-x}{T_2}\right) + m_1 \qquad (1)$$

m_0 is a constant that corrects for the scaling factor of the integral and m1 is a constant that corrects for baseline issues. The T2 measurements were done in triplicates on a Varian 600 MHz spectrometer equipped with a triple resonance cryogenic probe. The average T2 was calculated for further analysis.

2.2.3. Extrinsic Fluorescence Spectroscopy

Fluorescence measurements were conducted using Photon Technology International's (PTI) TimeMaster™ TM-2—LED lifetime strobe spectrofluorometer (Birmingham, New Jersey). Studies were performed at 25°C with a slit width of 2 nm and each spectrum was collected 4 times at a scan rate of 2 nm/sec. A 1 mM ANS stock solution was prepared. The stock's concentration was verified prior to the preparation of the final ANS concentration (50μM) for each fluorescence experiment. Concentrations of ANS stock solutions were determined using Solo VPE, using molar absorption coefficient $\varepsilon 350 = 4.95\, X\, 10^3\, M^{-1}cm^{-1}$.(Weber and Young, 1964)Stock solutions of BSA, A-ChytA, and B-LgA were prepared with the same buffer and filtered through a 0.22 μM filter. The final concentrations of protein were 0.0125 mg/ml, 0.025 mg/ml, 0.05 mg/ml, 0.1mg/ml, 0.2 mg/ml, 0.3 mg/ml and 0.4 mg/ml (except for BSA, which saturated the signal at this concentration). Samples of protein and ANS were made prior to measurement and stored in a dark place for 15 minutes covered with aluminum foil. Relative fluorescence intensities (RFI) of each solution (including buffer blank and buffer + probe blank) were measured. The RFI of protein blank samples (without ANS) were also prepared for the same concentrations. The net relative fluorescence intensities were obtained by subtracting the protein blanks (without ANS) from the protein samples that contained ANS. Measurements were done in duplicate. A fluorescence emission spectrum was recorded from 400nm to 650 nm for all proteins and an excitation wavelength of 375 nm was selected. Similarly to what has been put forward by [4], the surface hydrophobicity was determined from the protein concentration vs. fluorescence intensity at 470 nm plot and the initial slope, S0, is related to surface hydrophobicity [4].

2.2.4. Hydrophobic Interaction Chromatography

HIC was used with an in-line UV detector at 280 nm. 1mg/ml protein solutions were prepared in 20mM ionic strength sodium phosphate buffer (pH 6.95-7.05) and were injected into a high salt mobile phase of 20 mM sodium phosphate buffer with 1 M ammonium sulfate (pH 6.95-7.05). The pH of both buffers was adjusted with NaOH to maintain a pH of 7. The column was equilibrated with 100% 1.0 M ammonium sulfate in sodium phosphate buffer until a stable baseline was reached. Three proteins, BSA, B-LgA and A-ChytA, were injected separately into the column with an injection volume of 100 μL. Elution was accomplished by a 30-minute linear gradient from 100% 1.0 M to 0.0 M ammonium sulfate buffer at a flow rate of 0.5 ml/min. Each sample was filtered with a 0.22 μM filter and injected in triplicates.

3. Results and Discussion

3.1. Investigation of Hydrophobicity by Fluorescence and Hydrophobic Interaction Chromatography

We have first established relative hydrophobicity's for the three proteins of choice by employing traditional methods, fluorescence spectroscopy and hydrophobic interaction chromatography. Using the extrinsic fluorescence probe ANS, the surface hydrophobicity (S0) was measured for the three proteins and results are

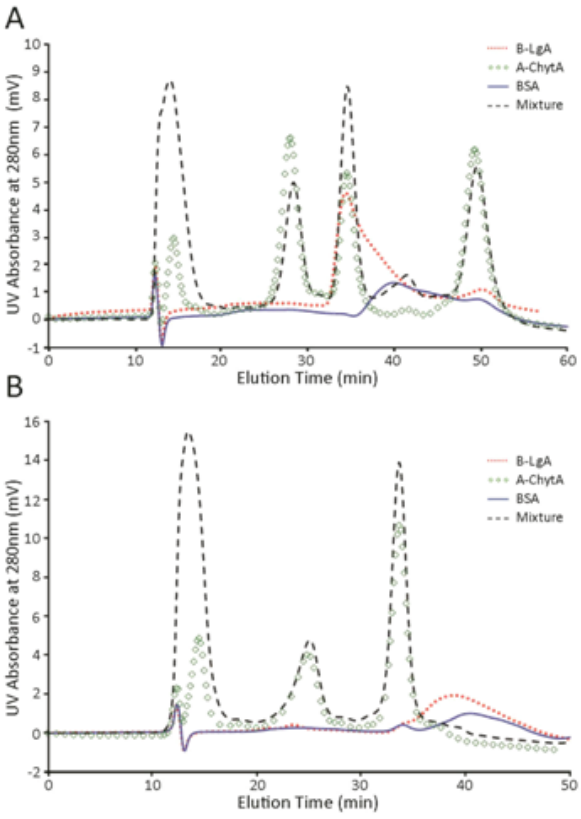
shown in Table 1. The S0 for BSA was 100-fold higher than the S0 for B-LgA and more than 500-fold higher than the S0 for A-ChytA. A limitation of using ANS is that it is negatively charged in solution, signifying that the interaction between the dye and protein is not only due to hydrophobic interactions. The S0 value measured for BSA at pH 7 at a low ionic strength of 15 mM, will give a different value if measured at high ionic strength conditions. As the ionic strength is increased, the charge on ANS will be screened and therefore interact differently with the protein. The charges on the surface of BSA will also be screened as the ionic strength is increased and therefore may produce a different hydrophobic So value, as has been shown previously [19]. The pH of the solution can also affect the local environment of the protein surface and its interaction with a charge probe. Since the two types of interactions, electrostatic and hydrophobic cannot be separated the fluorescence data from common ANS probe cannot give a reliable quantitative measure of hydrophobicity. Moreover, due to the fact that both the surface and the probe are influenced by solution conditions (i.e. pH, ionic strength etc.), the initial slope method, which produces an estimation of surface hydrophobicity, only tells the hydrophobic character of a protein at particular conditions. Therefore, these values should be used with caution when comparing proteins at different solution conditions and with different extrinsic dyes.

Table 1: Initial Slopes (S0) of ANS Bound to BSA, B-LgA and A-ChytA

Protein	Surface Hydrophobicity (S0)
BSA	1516
B-LgA	15.4
A-ChytA	2.8

HIC was next applied to determine the surface hydrophobicity of the three proteins. A butyl column was used for hydrophobic aliphatic interactions, and, separately, an aromatic phenyl column was used for π-π interactions between the phenyl ring and aromatic residues. A more hydrophobic protein will elute from the column at longer times. Figure 1 shows the results for the three proteins injected separately and as a mixture in two different columns. The order of hydrophobicity is similar for both columns. A-ChytA elutes off the column first, followed by BLgA and BSA. This indicates that of the three proteins, BSA is most hydrophobic. Similar behavior was seen with the retention times of BSA and B-LgA using an HIC linear gradient method [23]. All three proteins have multiple peaks, which indicates heterogeneity in the sample based on molecular weight (fragments or aggregates) or different conformational species present in the sample. Multiple peaks can also relate to changes induced by different ammonium sulfate concentrations or the strength of the hydrophobic stationary column [24]. Ueberbacher and coworkers used ATR FTIR to show that at high isocratic concentrations of ammonium sulfate, BSA does change conformation after being bound to the butyl HP column [25]. At a high ammonium sulfate concentration, the change in conformation could promote BSA aggregates, which may have different binding strengths leading to the difference in retention. Ueberbacher also states that partially unfolded proteins have a difficult time eluting off of the column, which may be why we see BSA having very broad peaks on both the phenyl and butyl columns. Though our elution patterns for BSA is not very different between the two columns, BSA may undergo structural changes and/or aggregate, making it difficult to assess the aromatic and aliphatic interactions.

Figure 1: HIC Elution Profile of BSA, A-ChytA, B-LgA, and Mixture on (a) Phenyl HP (b) Butyl HP Column



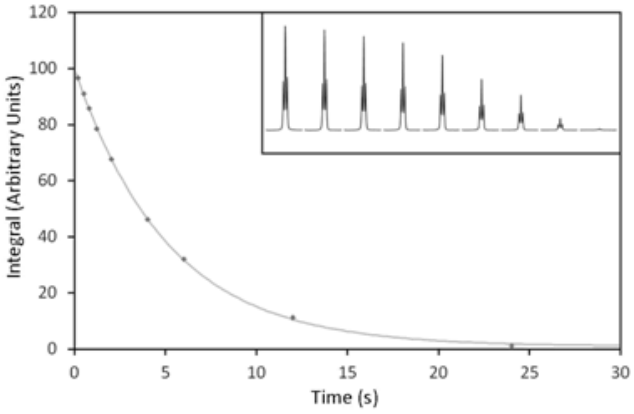
The elution profile for B-LgA is significantly different between the two columns, where the peak is relatively sharper for the phenyl column as well as having a small secondary peak eluting at a later time. This could be due to B-LgA having stronger π-π interactions. The least hydrophobic protein, A-ChytA has distinct peaks on both columns, having three peaks on the butyl column, and four peaks on the phenyl column. Multiple peaks present for A-ChytA may indicate numerous conformations at the pH and ionic strength studied as well as aromatic binding between these conformations to the phenyl column. These HIC results pose difficulties when trying to compare relative hydrophobicity's of these three proteins. Comparing the results from the phenyl HIC column to the fluorescence spectroscopy data, A-ChytA showed the least amount of binding to ANS even though they are similar in structure and may have equivalent binding mechanisms. At pH7 and low ionic strength solution conditions, A-ChytA has a high dipole moment, which could influence hydrophobic interactions to the charged probe ANS. At the solution conditions under which the HIC is carried out, these charged interactions are minimized which can lead to a change in the strength of hydrophobic interactions.

These results demonstrate that surface hydrophobicity is an interplay between both aliphatic and aromatic amino acids. It is also clear that because of the difference in solution conditions, which alter the amount of hydrophobic binding between techniques, finding additional information beyond the relative surface hydrophobicity is still a challenging task when utilizing established methods.

3.2. Optimization and Applicability of NMR for Evaluating Surface Hydrophobicity

In order to determine whether a small molecular weight probe interacts with a larger macromolecule target, such as protein or DNA, one can track changes in the probe relaxation rate through nuclear magnetic resonance (NMR) spectroscopy [26, 27, 28]. Large molecules are characterized by longer correlation times and relax faster in solution than small molecules. When small molecular weight probe binds to the protein, it relaxation rate increases, the phenomenon visually manifested by broadening of the probe peaks in corresponding NMR spectrum [29]. The parameter, which is defined by the relaxation rate of a molecule and is directly measurable from the NMR spectrum, is the linewidth of the molecules' corresponding peaks. The true line-width and T2, the transverse relaxation time, are related by the equation, $\vartheta_{1/2} = \frac{1}{\pi T_2}$ It is, thus, theoretically plausible to use either linewidth or T2 to monitor the binding of the probe to the protein. However in practice the measured line-width is affected by artifacts, $\vartheta_{actual} = \vartheta_{1/2} + \vartheta_{non-\text{hom}}$ The large errors in line-width measurements are associated with magnetic field non-homogeneity and are caused by difficulties in achieving exactly the same shims between samples. From this perspective, measuring T2, which is inversely proportional to the transverse relaxation rate, R2, is a better choice as it minimizes the problem of magnetic field inhomogeneity [30]. The T2 relaxation is determined by fitting the data points in time array to equation 1 as exemplified in Figure 2.

Figure 2: A Sample Exponential Decay (of 3 mM phenol) obtained from the CMPG-T2 Experiment. The Exponential is Fit with the Equation 1. The Variables of the Fit were m0 = 99.2, m1=0.88, and t2=5.147 s. The Inset Depicts the Peak of Interest (Triplet at 7.34ppm) at Consequent Time Points in the Array



There are two possible methods that can be used to observe the exponential decay of the peak of interest. It can be either done by monitoring the intensity of the peak at a certain chemical shift or by taking the integral of a defined region. There are two major benefits of using integral over intensity. First, integrals are less error prone because random noise in the defined region will be predominantly canceled out. Second, intensity is more sensitive to a number of factors not particularly related to binding, such as the line shape and possible chemical shift changes due to temperature instability [31]. Though integrals are more

favorable to use over intensities, the problems associated with overlapping peaks could still occur, especially in the case of broad protein peaks located underneath the sharp probe peaks. Integration over that region would combine the areas of both, the signal of interest and unwanted peaks, which may cause difficulties with fitting the data to a single exponential function and results in inaccurate T2 measurements. It is necessary to do protein peaks subtraction before fitting for such cases where significant overlap is visible and it is not possible to choose probe peaks from other nonoverlapping chemical shifts regions.

Our experiments were designed to determine the aliphatic and aromatic contributions to surface hydrophobicity. To do that, we have chosen a number of different small probes composed predominantly of either aliphatic or aromatic functional groups and have studied the degree of their association with three target proteins. The measurements were performed by following the changes in the relaxation properties of the probes upon binding to the targets. The reasoning for these experiments are twofold: i) the hydrophobic surface area of the protein could be quantitated by a number of weak interactions with small hydrophobic probes in solution, and ii) the measured contribution of the pure aliphatic interactions to the surface hydrophobicity might differ from the one measured with the probes containing aromatic component. We first tested tbutyl and phenol as the representatives of each class respectively in their ability to bind to BSA, A-ChytA, or BLgA over a wide range of the probe to the protein ratios. Because we expected weak non-specific interactions to take place (with Kds, dissociation constants, in high mM range), we wanted to make sure that our measurements are sensitive enough to reflect the ligand/receptor kinetics even at this low affinity limit. To simplify matters, we assume that the equilibrium between the free probe and the probe bound to the protein is in fast exchange. This is a reasonable assumption due to weak non-specific nature of the interactions in question [28]. Thus, the peaks observed correspond to a population average of the two states in fast exchange, the free probe in solution ([S]) and the one bound to a target protein ([SP]). The averaged peak would then have a relaxation rate that is weighted summation of the free and bound states as shown in the equation 2 below.

$$\frac{1}{T_{2obs}} = f_b \frac{1}{T_{2b}} + (1 - f_b) \frac{1}{T_{2\,free}} \text{ where } f_b = \frac{[SP]}{[S]_{total}} \qquad (2)$$

The T_{2obs} is the measured relaxation time of the probe mixed with protein in solutions, T_{2f} is the relaxation time of the probe alone, T_{2b} is the relaxation time of the probe in its bound state, and f_b is the fraction of probe bound. To simplify things, as a reasonable first approximation, T_{2b} is considered to be a constant that is equal to the relaxation time of the protein itself. The ^1H transverse relaxation times of the proteins were measured and the values used are 0.030 s, 0.020 s, and 0.003 s for B-LgA, A-ChytA, and BSA, respectively. Solving equation 2 for f_b results:

$$f_b = \frac{T_{2b}\left(T_{2f} - T_{2obs}\right)}{T_{2obs}\left(T_{2f} - T_{2b}\right)} \qquad (3)$$

When we consider nonspecific interactions of our probe to the protein, the probe is likely to bind to multiple sites. To simplify matters, we can assume that there are *n* binding sites and that each binding site is equivalent (which may not necessarily be true):

$$\frac{[SP]}{[P_o]} = n\left(\frac{[S]}{[S + K_d]}\right) \qquad (4)$$

Rearranging equation 4, we can obtain the model equation shown below.

$$f_b = \alpha - \sqrt{\left(\alpha^2 - \beta\right)} \qquad (5)$$

Where

$$\alpha = \frac{[S]_o + n[P]_o + K_d}{2[S]_o} \qquad (6)$$

And

$$\beta = \frac{n[P]_o}{[S]_o} \qquad (7)$$

Given that the concentration of the probe and protein are known, it is possible to fit the equation to obtain *Kd* and *n*. However, these values are highly dependent on how the fitting is done [32], subjecting this approach to data over-interpretation due to the assumptions made, multiple variables and the errors propagate. As an alternative more

intuitive approach, we plotted the bound probe fractions with respect to the overall concentrations of target protein (Figure 3), where the steepness of the linear fit can be considered as semi-quantitative measurement of protein hydrophobicity. Steeper slopes are correlated to a larger degree of bound probe compared to a shallow slope. We can, therefore, conclude that BSA is the most hydrophobic whereas A-ChytA (from the fresh preparation) and B-LgA are similarly less hydrophobic based on phenol probe, consistent with the outcomes from HIP and fluorescence spectroscopy. *Tert*-butyl alcohol has appreciably less affinity for BSA than phenol and exhibited no significant interaction with either A-ChytA or B-LgA.

Figure 3: Plot Showing the Fraction Bound of Probe vs the Concentration of Protein. The Probe: Protein Mixtures are Labeled and a Linear Fit of each Data set is shown. Two Sets of Data are Shown for Phenol:A-ChytA to Better Represent the Variability of T2s Obtained, Which is Due to Protein Degradation/Activation at Different Time Points of Protein Preparation

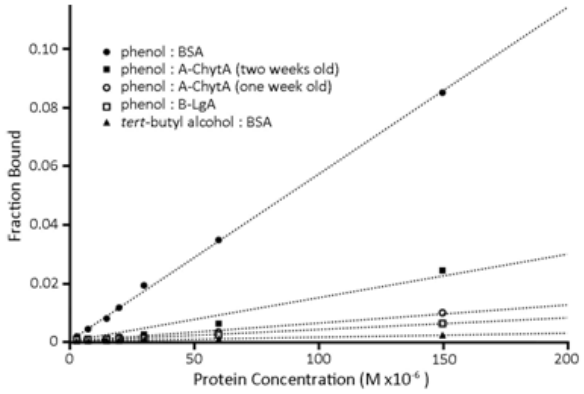


Figure 3 also illuminates the inconsistency of T2 measurements for A-ChytA, where it could be more or less hydrophobic depending on the sample preparation (two time points are presented as an example). We were quite surprised by this finding and have further investigated A-ChytA behavior in solution. We have found that these inconsistencies are due to the propensity of AChytA to degrade and/or oligomerize over time (see supplemental figure for time dependency), which could be potentially related to the autocatalysis from trace impurities of chymotrypsin [33]. Therefore, although unexpectedly for A-ChytA, which we have chosen as a reference protein assuming it reasonably good stability based on a literature search, variations in the T2 values determined by NMR can be used as an indicator for changes in sample integrity.

Table 2: Percent Reduction in T2 Relaxation Times of the Probes upon Binding to the Proteins

ProteinB-LgA Probe	**BSA**	**A-ChytA**	**B-LgA**
Tert-butyl alcohol	21	NB	NB
1-butanol	73	NB	NB
1-propanol	60	NB	NB
Phenol	97	11	18
Capped Amino Acids			
Leucine	58	9	NB
Valine	17	NB	NB
Phenylalanine	84	NB	8
Tryptophan	94	21	19
Tyrosine	79	6	NB

The Value shown above are the Percent change in T2 when Compared to the T2 of the free Probe Using the Average T2 Value Obtained, where percent change 100* /. Percent changes of less than 5% were within experimental error and are denoted with NB (no binding). The ratio for protein:probe is 1:50, where the protein concentration is 0.03 mM and the probe is 1.5 mM. T2 of the free probes and integration regions are: *tert*-butyl alcohol = 2.32s (1.28-1.18ppm), 1-butanol = 2.69 s (0.86-0.6 ppm), 1-propanol = 3.3 2s (0.86-0.6 ppm), phenol = 5.16 s (7.4-7.26 ppm), leucine = 0.67 s (0.84-0.7 ppm), valine = 0.83 s (0.95-0.6 ppm), phenylalanine = 2.04 s (7.28- 7.08 ppm), tryptophan = 2.20 s (7.56-7.45 ppm), tyrosine = 1.46 s (7.14-6.86 ppm).

Furthermore, to address the applicability of different small molecules as the hydrophobicity probe, the interactions with the same protein can be studied at one particular ratio to various probes, rather than performing a more extensive concentration-dependent titrations (as presented in Figure 3). This approach requires a sufficient amount of probe bound, so that more confidence can be attributes to the measured differences in the T2 (and correlated fraction bound values). For this, we have chosen a ratio 1:50 (from the titration curves for the phenol and tert-butyl) and the data for various probes is highlighted in Table 2. Instead of showing the absolute T2 values of the probes we present the percent reductions upon binding. This approach is taken to make analysis more intuitive: as the each probe has its own unique T2 value, it is difficult to compare the changes between probes looking just at the raw numbers. The choice of the probes tested and the outcome of the corresponding binding is discussed below.

Our experiments were designed to determine the aliphatic and aromatic contribution to hydrophobicity. According to the data presented in Table 2, non-specific binding of aliphatic and aromatic probes to the hydrophobic surface

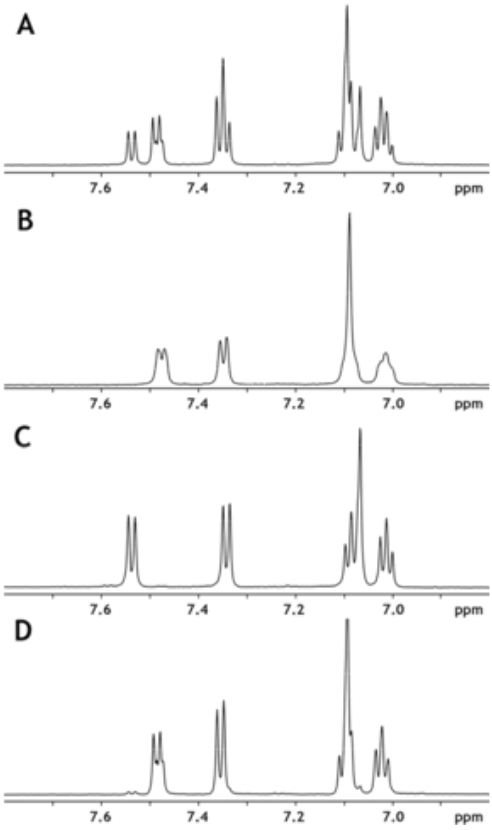
areas of tested proteins is not equivalent. When comparing the free probe to BSA-bound, there is a noticeable drop in T2 values for all aliphatic (tert-butyl, butanol, propanol) and aromatic (phenol) probes tested. However, these changes are significantly more pronounced for the aromatic probe as compared to the aliphatic ones: the most significant change occurs for phenol, 97% decrease, while changes for the aliphatic probes range from 21% in tert-butyl to 73% in 1-butanol. Additionally, small aliphatic probes do not show any significant binding to A-ChytA or B-LgA as the observed changes in T2 values are within measurement error. This is different in the case of phenol, which showed observable binding to both proteins.

Aliphatic vs. Aromatic Capped Amino Acids

To better represent protein-protein interactions, we decided to test natural amino acids with aliphatic and aromatic side-chains. As amino acids are typically charged in solution under physiological conditions and our goal was to study hydrophobic interactions, we selected several capped modifications to eliminate the charges and minimize electrostatic interactions. In general, the results were analogous to our findings with the small molecule probes. The aliphatic amino acids Leu and Val exhibited no binding to A-ChytA and B-LgA, while showing minor T2 depression in the presence of BSA. The 9% decrease for Leu is attributed to its low T2 (0.67 s alone and 0.61 s and in the presence of A-ChytA). On the other hand, we were able to observe noticeable drop in T2 values with aromatic Trp, expressing its binding to all three proteins.

Surprisingly, Tyr and Phe did not display expected binding, which made us question the applicability of capped amino acids as reliable binding probes. Although Tyr and Phe side chain moieties are highly analogous to phenol, the capped versions of these amino acids did not bind the same way phenol did. Since the side chains are most likely not the factor, we hypothesize that the capped C- and N-terminus are responsible. The cause may be due to increased bulkiness, resulting in steric hindrance. We also found out that different conformations of capped amino acids exist in solution. Generally, the different conformations of natural amino acids are readily interconverted in solution, so the corresponding peaks in 1H NMR spectrum represent an average of the conformations. We have found that capped amino acids have different conformations in slow exchange, leading to two (or, in some cases, even more) distinct conformations of non-equal populations.

Figure 4: Capped-Trp Spectra are Shown Zoomed on the Aromatic region. The Concentrations of Trp (shown for Trp alone in A) are 3 mM and the Ratio of Trp to Proteins, (shown for BSA in B, for A-ChytA in C, and for B- LgA in D), is 1:100. Integration Areas are: 7.56-7.45ppm = 0.99, 7.4-7.3ppm = 1.02, 7.15-7.06ppm = 2.0, 7.05-6.98ppm = 0.99. This Correlates to the Five Aromatic protons of Trp



As an example, the spectra of Trp alone and bound to different proteins are shown in Figure 4. Since Trp has only five unique aromatic hydrogens, there are different conformations of cappedTrp in solution as manifested by a more complex than expected pattern of peaks in the aromatic region (Figure 4A). This is further complicated by the disappearance of particular conformations in protein/probe solutions. By looking at the left most hydrogen (Figure 4), the left doublet at 7.54 ppm disappears in presence of BSA and partially disappears with B-LgA, but remains with AChytA. Conversely, the right doublet at 7.48 ppm disappears in presence of A-ChytA, but not BSA or B-LgA.

This feature is highly undesirable because different conformations complicate the exponential fit as they might have different binding capacity to the target protein and could no longer be fitted by a single exponential. For example, taking the integral over the range from 7.56 ppm to 7.45 ppm to examine the T2 of one hydrogen would not be appropriate for analysis as the observed peaks correspond to two or three unique hydrogens, some of which can totally disappear either due to the shift in equilibrium between conformations or due to line-broadening beyond detection upon tight binding. Thus, T2 values defined from these experiments become questionable.

Though this interaction-based conformational heterogeneity could be an interesting observation on its own in terms of underlying kinetics, it serves little purpose to our goals. Based on this, we concluded that Trp is not a reasonable probe to use. Furthermore, different conformations were also observed for capped Leu and Tyr, but not for Phe and Val. Though Phe and Val do not explicitly show different conformations in the NMR spectra, we are still hesitant on using those probes for future studies as the presence of the capped ends may still result in potential steric clashes. Thus, it is better to avoid larger molecules like capped amino acids as they may bring unnecessary complications to the analysis.

Comparison of Fluorescence, HIC, and NMR

In pharmaceutical protein formulations, aggregation, precipitation and opalescence are critical issues. It is recognized that protein molecules will have a combination of various interactions, some of which can be modulated by pH and ionic strength of the solution. However, the mechanistic understanding of the role hydrophobicity plays in these phenomena still needs further clarification. Application of NMR serves as an attempt to provide new tools to better quantify the hydrophobic effect. In this study, three techniques have all been used in an orthogonal approach to characterize surface hydrophobicity of three different proteins. We have found that in addition to being capable to distinguish between aromatic and pure aliphatic components in hydrophobic interactions as expected, NMR was also the most sensitive to protein stability, as highlighted in the case of A-ChytA.

4. Conclusions

Solution NMR is a fast and robust technique that is able to investigate the interaction between small molecular probes and proteins. In our quest to define the hydrophobicity of proteins, we have found that there is a difference between the non-specific binding capability of aliphatic and aromatic probes. There was a significant drop in T2 values for phenol in every protein solution tested, whereas there were no signs of binding for any aliphatics tested to A-ChytA or B-LgA. The same observations of the preferable aromatic interactions were found to be the case for capped amino acids, where the aliphatics Leu and Val did not bind to A-ChytA or B-LgA, but Trp, an aromatic, did. Other two aromatics though, Phe and Tyr, interacted only with BSA, but not with A-ChytA or B-LgA, which we attributed to steric clashes with bulky capped ends. Therefore, the application of capped amino acids as hydrophobicity probes is not suggested for future studies despite the potential advantage of mimicking protein-protein interactions.

The reliability of the NMR for measuring the surface hydrophobicity was investigated by comparing it to two commonly used methods, HIC and extrinsic fluorescence spectroscopy. All techniques show that BSA is the most hydrophobic. Fluorescence data also suggests that A-ChytA is the less hydrophobic than B-LgA, although the difference in the arbitrary units between the two is modest, only about 5x, especially compared to a much bigger difference between A-ChytA and BSA of about 500x. Results from both NMR and HIC about relative hydrophobicity between AChytA and B-LgA are inconclusive. Though the findings presented here are promising, there are still remaining questions to be answered. For instance, further studies are needed to better characterize phenol as an optimal binding probe (or possibly find another small aromatic probe), to determine a more suitable method for quantification of surface hydrophobicity by NMR, and to understand how and when aliphatic probes bind. Nonetheless, we believe that we made the case showing that NMR is a sensitive technique for measuring the surface hydrophobicity of proteins, with a specific focus on differences between aromatic and aliphatic binding modes, and that this technique can be used for protein targets with pharmaceutical significance. **(**Figure S1, Figure for Graphical Abstract)

Figure S1: The Physical Stability of A-ChytA was Monitored as a Function of Time. UV Chromatograms (280nm) of A- ChytA at time t=0 and after 24, 48 and 60 hours Holding at Room Temperature. Samples were Analyzed at a Concentration of 5mg/mL of A-ChytA, 0.8mL/min Flow Rate and with a 100mM pH 7 Sodium Phosphate Buffer with a Total Ionic Strength of 200mM (due to Addition of Sodium Sulfate) used as the Mobile Phase. The Shift in Monomer Peak is Error due Manual Injection of the Sample

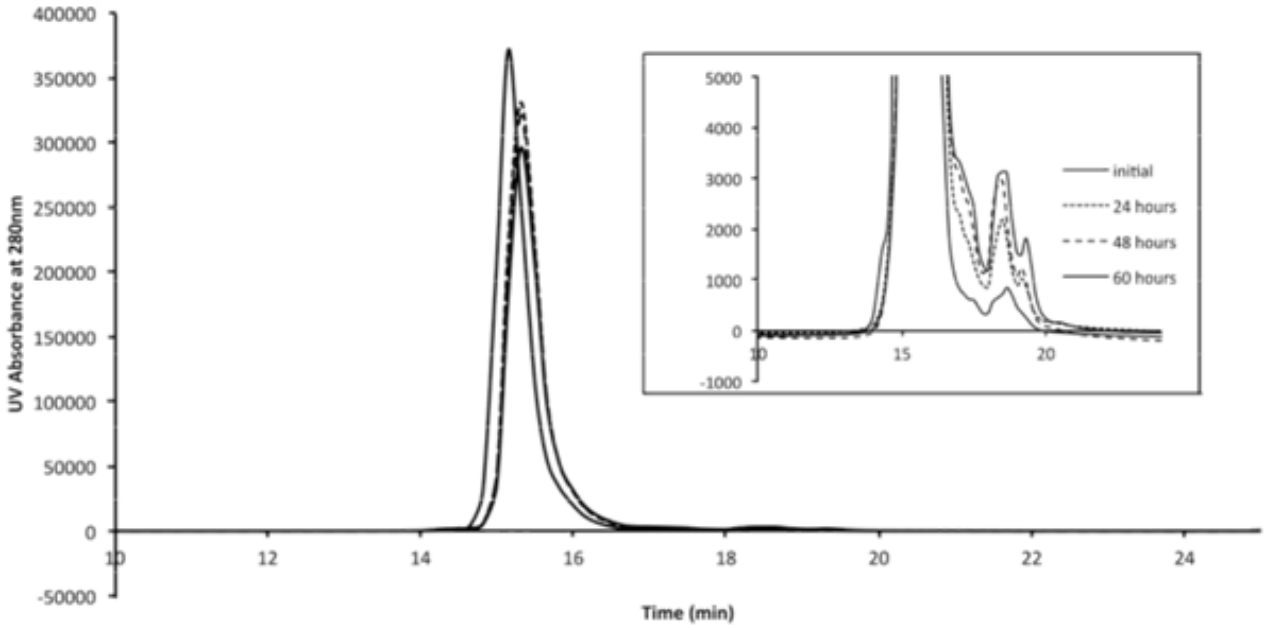
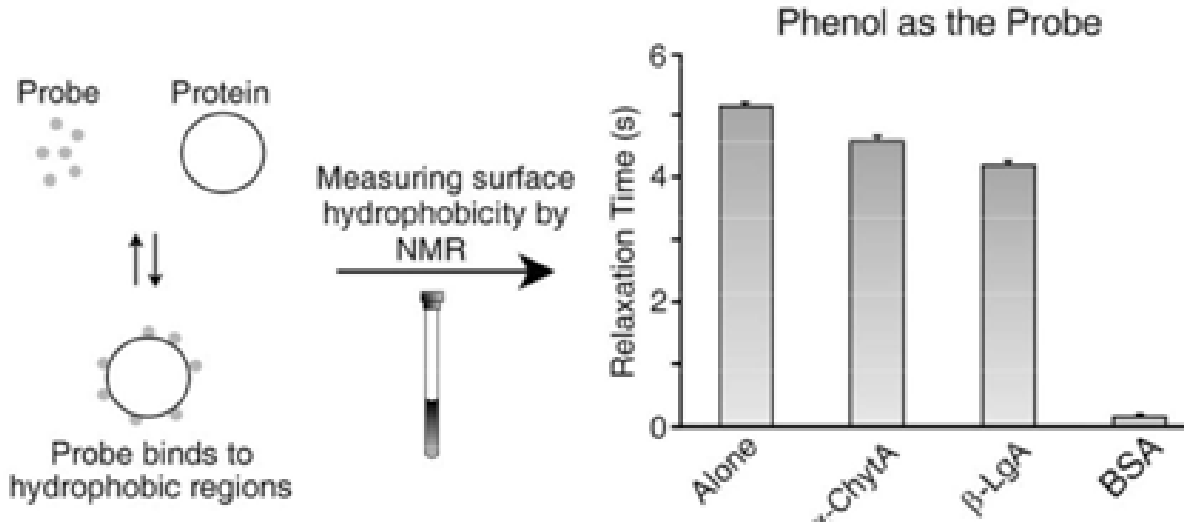


Figure for Graphical Abstract: Images show Hydrophobic Binding between Small Probe and Protein



Appendix A. Supplementary Data

The supplementary data includes the complete data set of T2 obtained at different molar ratios of probe: protein for both small molecule probes (Table S1) and capped amino acids (Table S2). The bigtau sets that are used to monitor the decay rate of the probe are listed in Table S3.

Acknowledgements

The authors would like to thank the PhRMA Foundation for financial support.

Table S1: Small Probes

Protein Probe	Alone	BSA	A-ChytA	B-LgA
Tert-butyl alcohol	2.32±0.01	1.84±0.01[C]	2.47±0.03[C]	2.31±0.02[C]
1-butanol	2.69±0.06	0.71±0.05	2.74±0.01[A]	2.74±0.10[A]
1-propanol	3.32±0.04	1.33±0.03	3.41±0.03[A]	3.25±0.01[A]
Phenol	5.16±0.03	0.16±0.01	4.60±0.07	4.21±0.04

A- This symbol denotes that protein subtraction was done to limit the error due to protein overlap.
B- This symbol denotes that the samples were run twice.
C- This symbol denotes that the samples were run once. Deviations shown are based on the deviation of the fit obtained from VnmrJ. All T2 values are presented in seconds. Aliphatic probes are shaded light grey and aromatic probes are shade grey. The errors presented for the T2 values are related to data acquisition and processing (same sample is evaluated multiple times), which shows the accuracy of determining the T2. This does not show the error that can occur due to sample preparation. All probe:protein samples are prepared at a 1:50 ratio (30 μM protein : 1.5 mM probe). For the probe's T2 in absence of protein, the concentrations used were 3 mM *tert*-butyl alcohol, 6 mM 1-butanol, 6 mM 1-propanol, and 3 mM phenol.

Table S2: Capped Amino Acid Probes

Protein Probe	Alone	BSA	A-ChytA	B-LgA
Leucine	0.67±0.01	0.28±0.01[B]	0.61±0.00[AB]	0.67±0.01[AB]
Valine	0.83±0.01	0.69±0.01[B]	0.86±0.00[AB]	0.86±0.00[AB]
Phenylalanine	2.04±0.01	0.32±0.00[B]	2.07±0.01[B]	1.87±0.01[B]
Tryptophan	2.20±0.03	0.13±0.01	1.71±0.01	1.79±0.02
Tyrosine	1.46±0.02	0.31±0.02	1.37±0.08	1.51±0.14

A- This symbol denotes that protein subtraction was done to limit the error due to protein overlap.
B- This symbol denotes that the samples were run twice.
ND refers to no data.
All T2 values are presented in seconds. Aliphatic probes are shaded light grey and aromatic probes are shade grey. The errors presented for the T2 values are related to data acquisition and processing (same sample is evaluated multiple times), which shows the accuracy of determining the T2. This does not show the error that can occur due to sample preparation. All protein-probe samples are prepared at a 1:50 ratio (30 μM protein : 1.5 mM probe). For the probe's T2 in absence of protein, the concentrations used were 3 mM for all probes, except Phe, which was 1.5 mM.

Table S3: Big Tau Sets

Probe	Protein	BigTau Set (s)
Tbutyl	None B-LgA A-ChytA	0.1,0.2,0.4,0.6,0.8,1.2,2,3,4,6,8,12,20
Tbutyl	BSA	0.1,0.2,0.4,0.8,1.2,2,3.5,6,8
Butanol	None BSA B-LgA A-ChytA	0.2,0.3,0.5,0.7,1.2,2,3.5,5.5,8,12
butanol	BSA	0.1,0.2,0.3,0.5,0.7,1.2,2,3,4.5,6
propanol	None B-LgA A-ChytA	0.2,0.4,0.6,0.8,1.2,3,6,9,12,20
propanol	BSA	0.1,0.2,0.3,0.5,0.7,1.2,2,3,4.5,6

Probe	Protein	BigTau Set (s)
phenol	None B-LgA A-ChytA	0.2,0.5,0.8,1.2,2,4,6,12,24
phenol	BSA	0.05,0.1,0.2,0.3,0.5,0.7,1.2,2,5
Leu	None B-LgA A-ChytA	0.1,0.2,0.4,0.8,1.2,2,4,8,16
Leu	BSA	0.05,0.1,0.2,0.3,0.5,0.7,1.2,2,5
Val	None BSA B-LgA A-ChytA	0.1,0.2,0.4,0.8,1.2,2,4,8,16
Phe	None B-LgA A-ChytA	0.1,0.2,0.4,0.8,1.2,2,4,8,16
Phe	BSA	0.05,0.1,0.2,0.3,0.5,0.7,1.2,2,5
Trp	None B-LgA A-ChytA	0.1,0.2,0.4,0.8,1.2,2,4,8,16
Trp	BSA	0.05,0.1,0.2,0.3,0.5,0.7,1.2,2,5
Tyr	None B-LgA A-ChytA	0.1,0.2,0.4,0.8,1.2,2,4,8,16
Tyr	BSA	0.05,0.1,0.2,0.3,0.5,0.7,1.2,2,5

Note that the minimum number of points used was 9 and that the differing ratios (1:25, 1:50, 1:100) all used the same bigtau set. The above table indicates the combination of protein and probe and their respective bigtau set

References

1. Kauzmann W (1979) Some Factors in the Interpretation of Protein Denaturation. 14: 1–63.

2. Tanford C (1962) Contribution of Hydrophobic Interactions to the Stability of the Globular Conformation of Proteins 84: 4240–4247.

3. Bigelow CC (1967) On the average hydrophobicity of proteins and the relation between it and protein structure. ℐ Theor Biol 16: 187–211.

4. Kato A, Nakai S (1980) Hydrophobicity determined by a fluorescent probe method and its correlation with surface properties of proteins. Biochim Biophys Acta Protein Struct 624: 13–20.

5. Amin S, Barnett GV, Pathak JA, et al. (2014) Protein aggregation, particle formation, characterization and rheology. Curr Opin Colloid Interface Sci 19: 438–449.

6. Haynes CA, Norde W (1994) Globular proteins at solid/liquid interfaces. Colloids Surf B Biointerfaces 2: 517–566.

7. Wang W (1999) Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int J Pharm 185: 129–188.

8. Cardamone M, Puri NK (1992) Spectrofluorimetric assessment of the surface hyrophobicity of proteins. Biochem J 282: 589–593.

9. Rassi El Z (1996) Recent progress in reversed-phase and hydrophobic interaction chromatography of carbohydrate species. J Chromatogr 720: 93–118.

10 Roettger BF, Ladisch MR (1989) Hydrophobic interaction chromatography. Biotechnol Adv 7: 15–29.

11. Fausnaugh JL, Regnier FE (1986) Solute and Mobile Phase Contributions to Retention in Hydrophobic Interaction Chromatography of Proteins. J Chromatogr 131–146.

12. Goheen SC, Gibbins BM (2000) Protein losses in ion-exchange and hydrophobic interaction high-performance liquid chromatography. J Chromatogr 890: 73–80.

13. Mahn A, Lienqueo ME, Asenjo JA (2007) Optimal operation conditions for protein separation in hydrophobic interaction chromatography. J Chromatogr B 849: 236–242.

14. Melander and Horváth (1977)

15. Queiroz JA, Tomaz CT, Cabral JMS (2001) Hydrophobic interaction chromatography of proteins. J Biotechnol 87: 143–159.

16. Rassi ZE, Horváth C (1986) Hydrophobic Interaction Chromatography of t-RNA's and Proteins. J Liq Chromatogr 9: 3245–3268.

17. Hawe A, Sutter M, Jiskoot W (2008) Extrinsic Fluorescent Dyes as Tools for Protein Characterization. Pharm Res 25: 1487–1499.

18. Alizadeh-Pasdar N, Li-Chan ECY, Nakai S (2004) FT-Raman Spectroscopy, Fluorescent Probe, and Solvent Accessibility Study of Egg and Milk Proteins. J Agric Food Chem 52: 5277–5283.

19. Haskard CA, Li-Chan ECY (1998) Hydrophobicity of Bovine Serum Albumin and Ovalbumin Determined Using Uncharged (PRODAN) and Anionic (ANS-) Fluorescent Probes. J Agric Food Chem 46: 2671–2677.

20. Pellecchia M, Sem DS, Wüthrich K (2002) NMR in drug discovery. Nat Rev Drug Discov.

21. Meiboom S, Gill D (1958) Modified Spin-Echo Method for Measuring Nuclear Relaxation Times. Rev Sci Instrum 29: 688.

22. Aguilar JA, Nilsson M, Bodenhausen G, et al. (2012) Spin echo NMR spectra without J modulation. Chem Commun 48: 811–813.

23. Hachem F, Andrews BA, Asenjo JA (1996) Hydrophobic partitioning of proteins in aqueous two-phase systems. Enzyme Microb Technol 19: 507–517.

24. Wu SL, Figueroa A, Karger BL (1986) Protein Conformational Effects in Hydrophobic Interaction Chromatography. J Chromatogr 371: 3–27.

25. Ueberbacher R, Haimer E, Hahn R, et al. (2008) Hydrophobic interaction chromatography of proteins. J Chromatogr 1198-1199, 154–163.

26. Dubois BW, Evers AS (1992) Fluorine-19 NMR spin-spin relaxation (T2) method for characterizing volatile anesthetic binding to proteins. Analysis of isoflurane binding to serum albumin. Biochem 31: 7069–7076.

27. Fielding L (2007) NMR methods for the determination of protein–ligand dissociation constants. Prog Nucl Magn Reson Spectrosc 51: 219–242.

28. Lepre CA, Moore JM, Peng JW (2004) Theory and Applications of NMR-Based Screening in Pharmaceutical Research. Chem Rev 104: 3641–3676.

29. Fischer JJ, Jardetzky O (1965) Nuclear Magnetic Relaxation Study of Intermolecular Complexes. The Mechanism of Penicillin Bindingto Serum Albumin. J Am Chem Soc 87: 3237–3244.

30. Hahn EL (1950) Spin echoes. Phys Rev.

31. Viles JH, Duggan BM, Zaborowki E, et al. (2001) Potential bias in NMR relaxation data introduced by peak intensity analysis and curve fitting methods. J Biomol NMR 21: 1–9.

32. Fielding L, Rutherford S, Fletcher D (2005) Determination of protein-ligand binding affinity by NMR: observations from serum albumin model systems. Magn Reson Chem 43: 463–470.

33. Velev OD, Kaler EW, Lenhoff AM (1998) Protein Interactions in Solution Characterized by Light and Neutron Scattering: Comparison of Lysozyme and Chymotrypsinogen. Biophys J 75: 2682–2697.

Citation: Olga Vinogradova (2018) NMR as a Semi-Quantitative Tool for Evaluating Protein Surface Hydrophobicity. SF Pharma J 1:2.