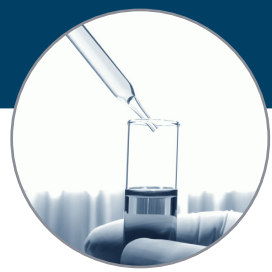

Research in bioanalysis and separations at the University of Nebraska – Lincoln

The Chemistry Department at the University of Nebraska – Lincoln (UNL) is located in Hamilton Hall on the main campus of UNL in Lincoln, NE, USA. This department houses the primary graduate and research program in chemistry in the state of Nebraska. This program includes the traditional fields of analytical chemistry, biochemistry, inorganic chemistry, organic chemistry and physical chemistry. However, this program also contains a great deal of multidisciplinary research in fields that range from bioanalytical and biophysical chemistry to nanomaterials, energy research, catalysis and computational chemistry. Current research in bioanalytical and biophysical chemistry at UNL includes work with separation methods such as HPLC and CE, as well as with techniques such as MS and LC–MS, NMR spectroscopy, electrochemical biosensors, scanning probe microscopy and laser spectroscopy. This article will discuss several of these areas, with an emphasis being placed on research in bioanalytical separations, binding assays and related fields.

High-performance affinity separations & chromatographic immunoassays

One area of active research at the University of Nebraska – Lincoln (UNL) is the development of novel separations for biological and environmental samples based on high-performance affinity chromatography (HPAC) and affinity CE (ACE). This work is carried out in the laboratory of David S Hage. Both HPAC and ACE are based on the use of a biologically related agent (e.g., an antibody, aptamer, receptor or transport protein) for the selective recognition and binding of a target analyte in a sample [1–4]. In HPAC, the selective binding agent is immobilized within a column and used as a stationary phase for analyte retention [1,2,4]. In ACE, the binding agent is combined with the sample or placed within the running buffer to bind to the target and alter its electrophoretic mobility, thereby leading to the separation of this target from other sample components [3]. The selective, strong interactions present in many biological systems help to make HPAC and ACE powerful methods for the rapid study of specific targets in complex samples. These methods can be used alone for the detection and measurement of analytes, or can be combined with other techniques to develop multidimensional schemes for separation and analysis (e.g., the use of immunoaffinity columns with reversed-phase LC or as part of an LC–MS system) [1–4]. A large number of routine and custom-designed HPLC systems are available for such work in the Hage laboratory and in the facilities within the Chemistry Department at UNL. A variety of detection formats are possible with these HPLC systems, including absorbance, fluorescence or MS and more specialized modes such as chemiluminescence or near-infrared fluorescence. Several CE systems are also available for use in this research with either absorbance or fluorescence detection.

A specific topic area of emphasis in the Hage laboratory is the use of antibodies and other selective binding agents in HPLC systems to produce 'chromatographic immunoassays' [5,6]. It has been demonstrated in the past that these methods can be used for the rapid and selective measurements of drugs, hormones, peptides, proteins and many other targets in biological samples. These properties make chromatographic immunoassays appealing for use in clinical testing, pharmaceutical analysis, biotechnology and environmental studies [5–15]. When used in combination with methods such as reversed-phase LC or CE, chromatographic immunoassays can also be adapted for the simultaneous analysis of several compounds within a given class of chemicals [5–9].

One application of Hage's work in this area has been the creation of an automated and portable system for the determination of triazine herbicides in environmental samples [7,8]. This method combined the use of a small antibody column with a reversed-phase HPLC column and absorbance detection. This approach has been used in both the laboratory and in a portable device for field work. In work with the portable system, it was possible to provide results at the site of a river or stream within 10 min of sample injection. This approach has been used not only for triazine herbicides, such as atrazine, but has been adapted for use with other environmental agents (e.g., chloroacetic acid herbicides such as 2,4-D) by changing the type of antibody column used in the system [7–9].

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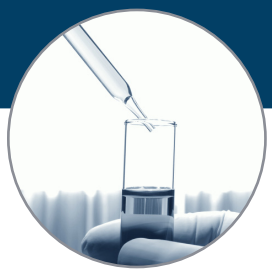


Other chromatographic immunoassays that have been developed by the Hage laboratory include techniques for measuring specific hormones, peptides, drugs and proteins in clinical samples [10–15]. These methods have been shown in numerous studies to be precise, easy to automate and to take only a small period of time to perform (i.e., typically only a few minutes). In addition, Hage's group has explored and developed many formats for use in these methods. These formats range from competitive binding assays to sandwich immunoassays, displacement immunoassays and one-site immunometric assays. As part of these studies, the Hage group has also been active in working with chromatographic theory and computer models to describe the response and behavior of these assays under various operating conditions [6,9,10,15].

A recent application of chromatographic immunoassays has been the development of ultra-fast immunoextraction and the use of this approach to measure the 'free fraction' of a drug or hormone in a biological sample (Figure 1) [10–12,16]. Ultrafast immunoextraction is based on the use of an affinity microcolumn with a small layer of antibodies that can bind quickly and selectively to the desired target. When used to measure the free fraction of a drug or hormone, this free fraction is extracted by the antibody column in a very short period of time (80–120 ms), which makes it possible to isolate this fraction without disturbing the fraction of the drug or hormone that is bound by other sample components. The results of this approach have been found to give good correlation with reference methods such as ultrafiltration. However, this information can be obtained with chromatographic immunoassays in much less analysis time than is required by other methods, making it an attractive approach for use in pharmacological studies and in clinical chemistry for personalized medicine [10–12].

Chiral separations & studies of biological interactions

Two other ways that HPAC and ACE can be used is for chiral separations or to study biological interactions (e.g., protein–protein binding or protein–drug binding). This research is of current interest for the high-throughput screening of drug candidates for a given protein and for predicting the behavior of drugs and proteins in the body by using separation systems as models for these biological systems [4,17–20].

One protein the Hage group has studied extensively with regard to chiral separations and drug–protein binding is human serum albumin (HSA). HSA is the most abundant protein in serum and plays an important role in delivering many types of drugs throughout the body. The Hage group has shown that HSA can be immobilized within HPLC columns and used in rapid chiral separations [21–24] or to study how this protein binds to various drugs, hormones and other small solutes in serum [21–32]. Chemicals that have been examined with these

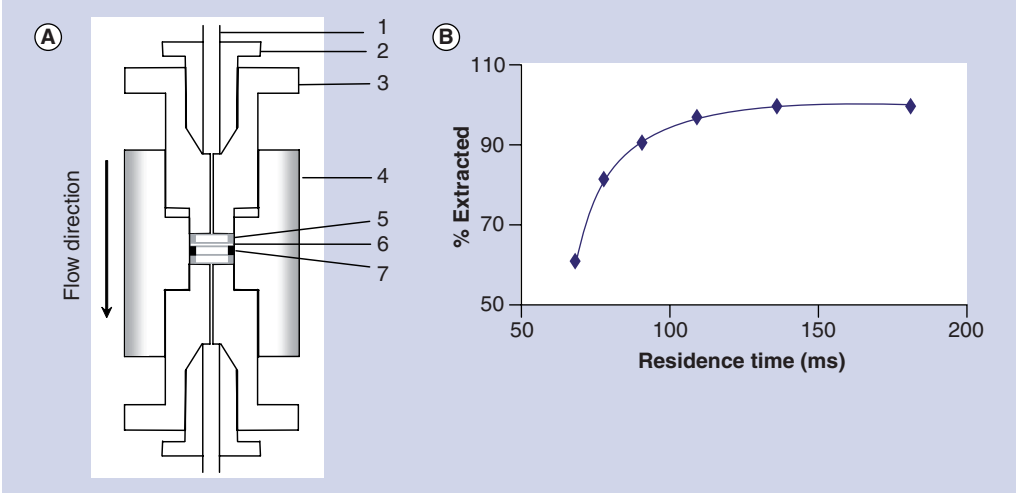
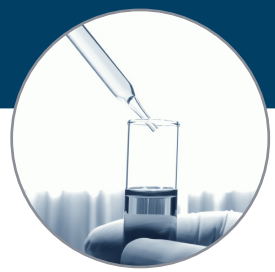


Figure 1. (A) General design of a small affinity column for use in ultrafast immunoextraction and (B) the extraction of fluorescein as a model target by immobilized antibodies within such a column. The plot in **(B)** indicates that 95% extraction of the target could be obtained in 100 ms by this approach. The components in **(A)** were as follows: (1) connection tubing, (2) fitting, (3) end fitting assembly, (4) column casing, (5) frit, (6) sample distributor, and (7) affinity column or affinity disk.
Adapted with permission from [16].



HSA columns include warfarin, thyroxine, tryptophan, ibuprofen, digitoxin, clomiphene, tamoxifen, phenytoin and carbamazepine. In addition, the Hage group has recently created columns that contained the serum protein α_1-acid glycoprotein (AGP) in a fully active form that can be used in chiral separations or binding studies (Figure 2) [33–36]. The group has also developed columns containing complex particles such as high-density lipoprotein and low-density lipoprotein for the investigation of drug interactions with these binding agents in serum [37,38].

As part of this work, the Hage group has developed several new approaches for examining the biological interactions by HPAC or ACE. Examples of these methods are approaches for the study of low-solubility drugs [28–30], site-selective studies of agents with multiple binding sites on a protein [25,31] and techniques for examining the interactions of drugs or solutes that have allosteric interactions on a protein [39]. A variety of methods for examining the kinetics of drug–protein interactions have also been created for use with HPAC [40–43], along with HPAC and ACE approaches for examining the interactions of drugs with the soluble form of a protein [10–12,19,20,44]. These approaches have been used with both native proteins and with modified proteins, as has recently been demonstrated in studies using HSA that has undergone non-enzymatic glycation [45–47]. Related work that has been associated with these efforts has involved the creation of affinity microcolumns, which can be used in binding studies with small amounts of proteins that have been obtained from individuals and that can be examined in detail by HPAC over the course of hundreds of experiments [45–49].

LC–MS, GC–MS & related methods

Research facilities are also available in the Chemistry Department for work in LC–MS and GC–MS within the Nebraska Center for Mass Spectrometry. The GC–MS system consists of an Agilent 6890N instrument with an auto-injection system and a Waters GCT TOF-MS for work with either electron ionization or chemical ionization. There are also LC–MS systems that use ESI, including Waters Q-TOF Ultima and Synapt G2 Q-TOF systems and a Thermo LCQ ion-trap mass spectrometer. Equipment for conducting MALDI-TOF-MS is also available in the center.

The group of Liangcheng Du makes use of many of these facilities as part of their research. The Du group is studying the molecular mechanisms by which a variety of organisms (e.g., bacteria, fungi and plants) make structurally complex and biologically active natural products. The goal of this work is to use this knowledge to produce new products through genetic engineering that will be beneficial to humans. A specific example of this work concerns the mechanism of biosynthesis for fungal polyketides. Polyketides are probably the most significant group of natural products in terms of their importance to human medicine. Most studies to date with these compounds have focused on polyketides from bacteria. Although fungi produce numerous polyketides, their biosynthetic mechanism remains largely unknown. The Du group has been studying mycotoxin fumonisins, which are produced by a number of economically significant fungi such as the pathogenic fungus *Fusarium verticillioides*, which is a widespread contaminant of corn and maize-derived food and feeds. The ingestion of fumonisin-contaminated corn causes fatal diseases in livestock and imposes a cancer risk to humans. By using HPLC, GC–MS and LC–MS, the Du group has identified multiple biosynthetic intermediates from various fungal strains, along with mutants that are generated by specifically changing the biosynthetic genes for fumonisins. This research has helped to reveal the pathway for the biosynthesis of fumonisin [50–52].

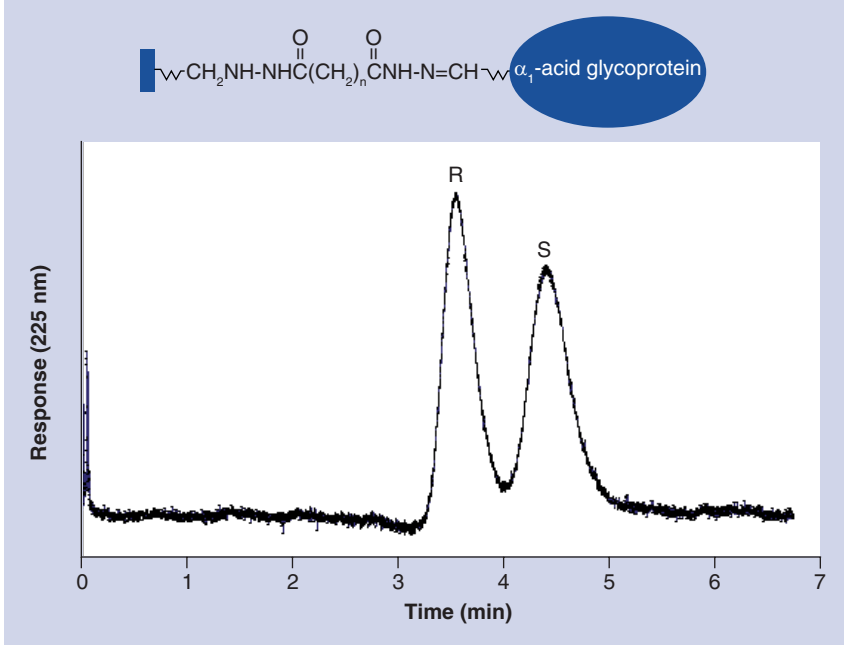
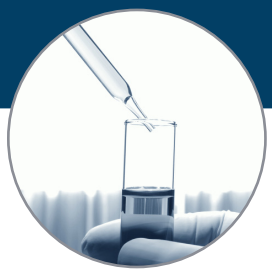


Figure 2. General structure of a silica support containing immobilized α_1-acid glycoprotein, and the use of this support in the chiral separation of (*R*)- and (*S*)-propranolol. The conditions used in this separation were as follows: mobile phase: pH 7.4; 0.067 M phosphate buffer with 2% (v/v) 2-propanol; temperature: 37°C; flow-rate: 2.00 ml/min.
Adapted with permission from [34].



In another project, Du has been studying the genetics and function of novel antifungal natural products such as **heat-stable antifungal factor** (HSAF; i.e., dihydromaltophilin). HSAF has been isolated from the biocontrol agent *Lysobacter enzymogenes* C3 [53]. This compound is a potent inhibitor of a wide range of fungi. The goals of the Du group in this area have been to develop novel antifungal drugs for humans and 'green' strategies for fungal disease control in agriculture. HPLC, MS and NMR have been used to determine the structure of HSAF, which has a unique macrocyclic lactam system that contains a tetramic acid moiety and a 5,5,6-tricyclic skeleton [54]. Du has also determined the biosynthetic genes for this compound and has identified several compounds that are produced by gene disruption and gene-replacement mutants [55].

The use of LC–MS or separation methods in combination with MALDI-TOF-MS is also of interest to the Hage group. For instance, the group is using binding studies based on HPAC to provide information on the function of a biological molecule and its interactions with other compounds, while MS is used to provide information on the structure of the biomolecule [56–59]. As an example, the group has used methods based on quantitative proteomics and MS to examine the structure of immobilized HSA on HPLC supports [57]. The general scheme that was used for this research is shown in Figure 3. In this process, the immobilized HSA was digested with a proteolytic enzyme in the presence of normal water, while a soluble form of the same protein was digested in ^{18}O-enriched water. After the digestion reactions were quenched, the soluble fractions of the digests were combined, fractionated and analyzed using MALDI-TOF-MS. This approach made it possible to identify the major immobilization sites for HSA and to determine how these immobilization sites might affect the binding of drugs to this protein in its immobilized form. A similar approach has been used to compare the modifications that occur on glycated HSA versus normal HSA and to examine the relationship between these modifications and the changes in drug–HSA interactions that can occur during diabetes [58,59].

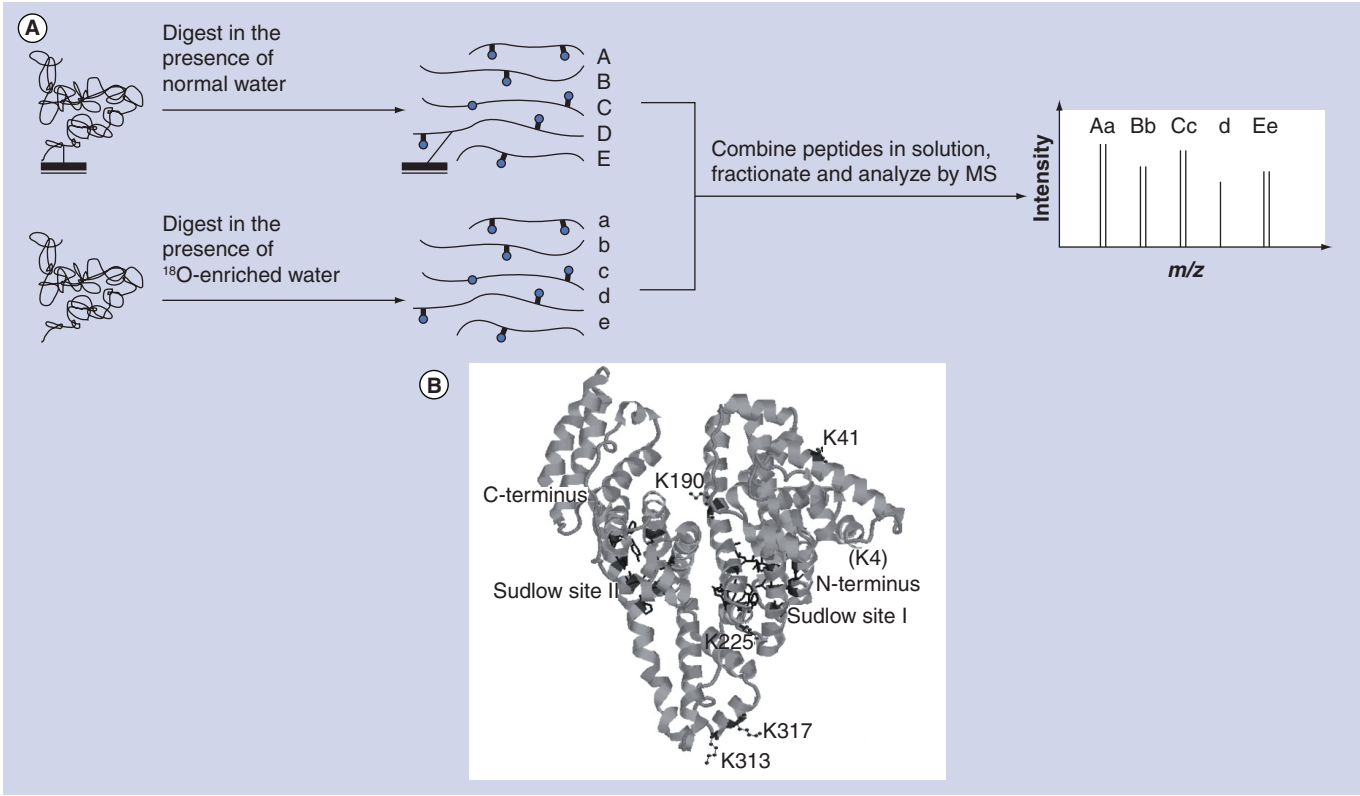
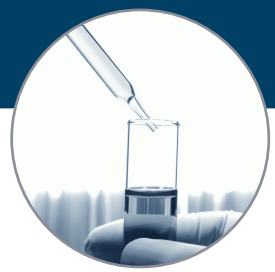


Figure 3. (A) The use of ^{18}O/^{16}O-labeling with quantitative proteomics and MS to examine the immobilization sites for a protein on a solid support and (B) the results that were obtained with this approach for human serum albumin that was immobilized to HPLC-grade silica by the Schiff base method. The results for human serum albumin show the most common residues found to be involved in the covalent immobilization of this protein. These results also show the locations of these residues when compared with the two main sites for drug interactions with human serum albumin (i.e., Sudlow sites I and II). Adapted with permission from [57].



Ion mobility MS

Another capability that has recently been added at UNL is the use of separations based on gas-phase ion mobility (IM) in conjunction with MS. This technique is the subject of research in the laboratory of Eric D Dodds. The equipment used for this work is a Waters Synapt G2 HDMS IM-enabled Q-TOF hybrid mass spectrometer. This instrument offers the high mass accuracy (error < 1 ppm) and high mass resolution ($m/\Delta m > 40{,}000$) of a state-of-the-art Q-TOF, as well as the ability to carry out MS/MS by means of collision-induced dissociation or electron transfer dissociation. The IM feature of the instrument further provides for the separation and collisional cross-section measurement of analyte ions. IM is well-suited for use with LC and TOF-MS because the timescale of an IM separation (10^{-3} s) lies between those for typical LC runs (10^{3} s) and TOF-MS (10^{-6} s). This means that IM can be used with LC and TOF-MS to provide an additional dimension of separation without increasing analysis time. The trade-off in combining these methods is a reduced duty cycle because ions must be gated to the IM device rather than continuously sampled as a beam by the TOF-MS.

An IM separation of isomeric peptides is shown in Figure 4. The two peptides shown in this example contain the same amino acids, and therefore have the same exact mass, but these peptides have their amino acids arranged in mirror sequences (i.e., SDGRG and GRGDS). This pair of reverse-sequence peptides has been previously studied via IM [60,61] and provides a convenient benchmark for determining IM resolution. In this example, the two peptides are nearly baseline resolved as their doubly protonated ions. Aside from the separation of isomers, IM fractionates ions of different charge states into separate regions of drift time versus *m/z* space. Similarly, biomolecules of different classes (e.g., lipids vs carbohydrates) are known to occupy distinct regions of this space [62]. This feature can be useful for targeting analytes of a particular class or charge state.

The unique capabilities of IM are being applied in the Dodds laboratory to address various questions concerning biomolecular structure. One area of application that is being examined is that of site-specific glycoproteomics. Among the many ways in which proteins are modified, glycosylation is uniquely complex and involves many levels of organization. Rather than being merely present or absent at a given site, glycosylation can lead to the possibility of site heterogeneity (e.g., different glycans occupying the same site on different copies of a protein), as well as structural isomerism within a given glycan composition. In conjunction with other separation and MS techniques, IM is being applied to address these complexities. A second field of study that is being considered is in the interrogation of quaternary architecture in noncovalent protein assemblies. As most proteins function as noncovalent complexes, an appreciation of subunit stoichiometry and complex topology is essential for an understanding of structure–function relationships. Condensed-phase and gas-phase chemical strategies, including IM, are being utilized to probe the structures of protein complexes that are sampled directly from physiological-like aqueous solutions. The objective of this work is to obtain information on subunit arrangement and interfaces in these systems.

NMR methods for ligand affinity screens & metabolomics

Complementary work in bioanalysis that is occurring at UNL is in the field of NMR spectroscopy and the use of this method to examine a variety of biological problems. NMR methods are currently being developed and used in the laboratory of Robert Powers to understand the structure, function and evolution of novel proteins and their corresponding therapeutic utility [63–65]. NMR is being employed in this work as part of a functional genomics effort to determine the

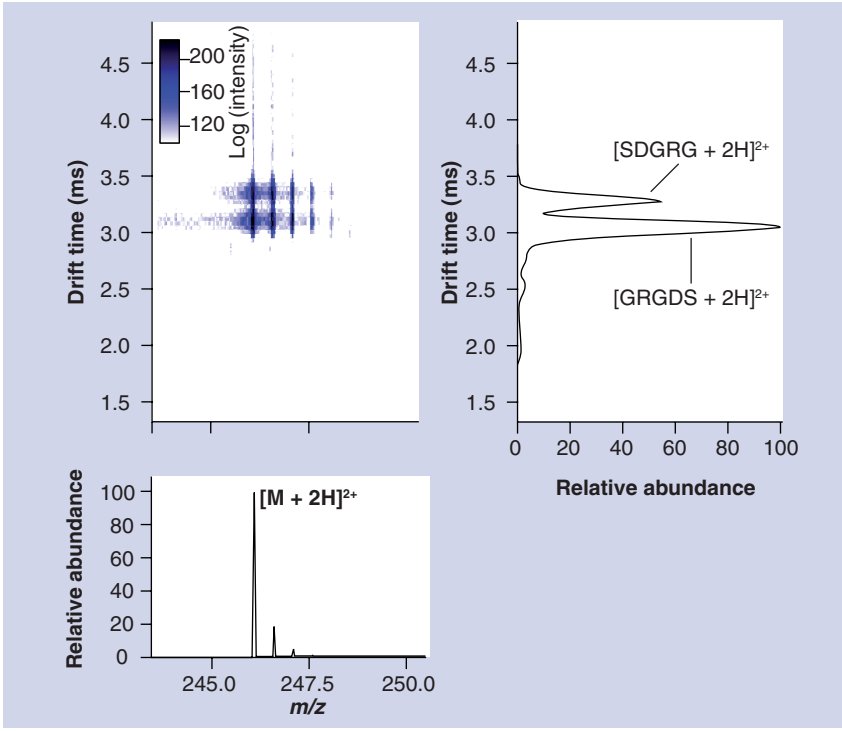
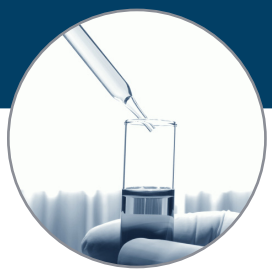


Figure 4. Ion mobility separation of the reverse sequence peptides SDGRG and GRGDS. The $[M+2H]^{2+}$ ions are observed at *m/z* 246.1 (monoisotopic).



structures of biomolecules, to investigate biomolecular and small-molecule interactions, to screen for functional ligands and potential drugs, and to monitor *in vivo* protein and drug activity.

These NMR studies are carried out using equipment in the Chemistry Department's Research Instrumentation Facility. This facility operates and maintains several NMR spectrometers, including a 600-MHz Bruker AVANCE spectrometer, which is a five-channel NMR system capable of working with both solids and liquids. This instrument has triple-axis gradients, enabling sophisticated gradient experiments involving multiple nuclei, full broadband capabilities and a variable temperature unit for performing high and low temperature experiments. This instrument is also equipped with a range of probes for use with liquids, including a TXI triple resonance $^{1}H/^{13}C/^{15}N$ probe optimized for protein experiments. A second NMR instrument that is available is a three-channel 500-MHz Bruker DRX NMR spectrometer with a variable temperature unit that features a 5 mm $^{1}H/^{13}C/^{15}N$ cryoprobe. This system excels at the direct detection of protons and as a platform for the indirect detection of ^{13}C or ^{15}N through 2D experiments. The addition of a Bruker BACS-120 sample changer to this instrument allows for the high-throughput screening of ligand-affinity interactions and work in metabolomics.

One method being developed in the Powers laboratory is functional annotation screening technology by NMR (FAST-NMR), as illustrated in Figure 5 [66,67]. This method combines structural biology and NMR ligand-affinity screens with bioinformatics to generate a functional hypothesis that is based on structural and sequence similarities in ligand-binding sites. FAST-NMR is similar in concept to sequence and structure homology. However, instead of relying

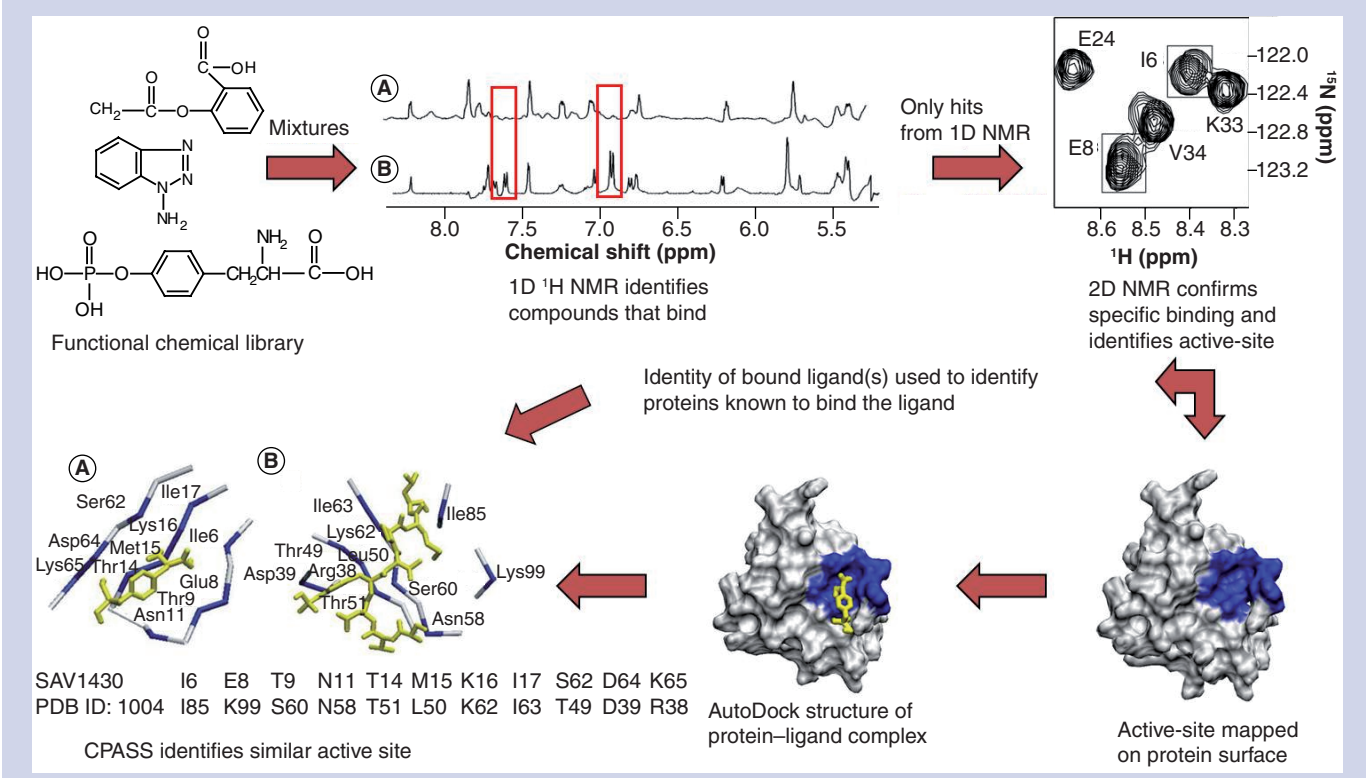
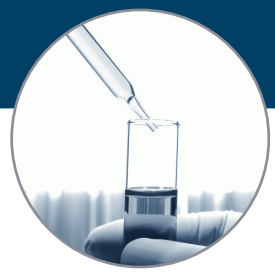


Figure 5. Flow chart for a functional annotation screening technology-NMR assay. Functionally uncharacterized proteins are first screened against mixtures of ligands from the functional chemical library. Reference 1D ^{1}H NMR spectra of the mixtures are then compared with those containing the protein, where a hit is identified by changes in NMR line width. Only the ligands identified as binding in the primary screen are further assayed in the secondary 2D ^{1}H–^{15}N HSQC NMR experiment. Chemical shift changes are used to confirm a specific interaction and to identify the binding site from mapping of the chemical shift perturbations on the protein's surface. The binding site and chemical shift perturbations are utilized to determine a rapid co-structure using AutoDock. This co-structure is then used by the CPASS to compare the ligand-defined binding site from the hypothetical protein to all other protein–ligand interactions present in the protein database. A general biological function can then be assigned based on an observed similarity to a ligand-defined binding site for a protein of known function.
CPASS: Comparison of Protein Active Site Structures.
Reproduced with permission from [67].



on global similarities, a functional assignment is made by focusing on evolutionary stable functional regions. FAST-NMR incorporates a multistep NMR ligand-affinity screening technique [68] with a rapid approach to generate protein–ligand co-structures [69] and a technique to measure dissociation constants from NMR screens [70], along with Comparison of Protein Active Site Structures and Protein Function, Evolution, Structure and Sequence as tools for bioinformatics [71,72]. Over 24 proteins have been examined by this FAST-NMR assay. These proteins have included *Staphylococcus aureus* protein SAV1430, *Pseudomonas aeruginosa* protein PA1324, *Pyrococcus horikoshii* OT3 protein PH1320, human protein Q13206, *Bacillus subtilis* protein YndB and *Salmonella typhimurium* PrgI protein [66,67,73,74]. Recent efforts with this approach have focused on the analysis of proteins of unknown function that are associated with pancreatic cancer.

NMR spectroscopy has also been used for differential metabolomics by the Powers group [75–78]. In this work, NMR is used to examine the metabolome of lyzed cells or body fluids and to study changes that result from the influence of disease, drug treatments or environmental stress. NMR combined with principal component analysis is used to measure global changes between cells under various conditions. Similarly, NMR combined with ^{13}C-labeled metabolites permits a detailed analysis of metabolite concentration changes. This differential NMR metabolomics methodology is currently being used to understand the biofilm of staphylococcus, to understand mechanisms of drug activity and resistance in tuberculosis, to identify biomarkers for multiple sclerosis, to improve the industrial-scale production of diphtheria toxin and to aid in drug discovery for pancreatic cancer.

Development of new supports & separation media for bioanalysis

One unique capability of researchers in the Chemistry Department at UNL is their ability to create, characterize and evaluate a variety of novel materials for use in separation methods. For

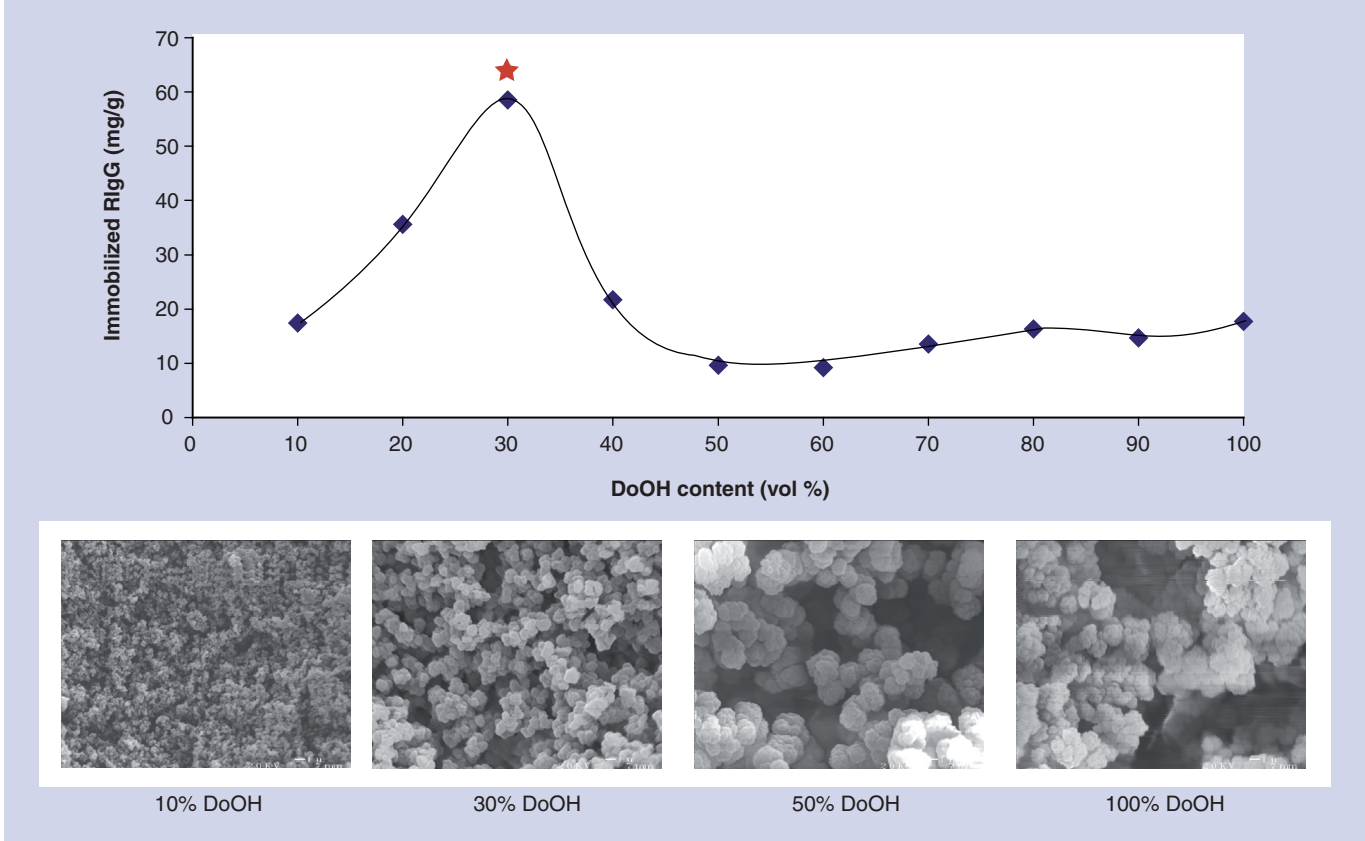
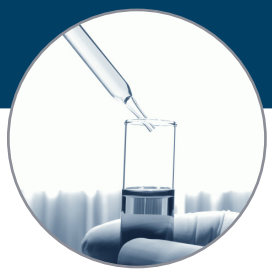


Figure 6. Effect of changing the relative amount of dodecanol in the porogen during monolith preparation on the amount of immobilized IgG-class antibodies that could be attached to poly(glycidyl methacrylate-co-ethylene dimethacrylate) (GMA/EDMA) monoliths. The star in the top diagram shows the conditions under which the maximum amount of immobilized IgG was obtained.
DoOH: Dodecanol.
Adapted with permission from [16].



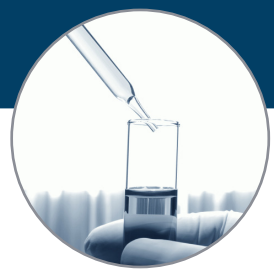
instance, Hage has been active for many years in the optimization and creation of new schemes for immobilizing and modifying biological agents for use in HPAC or other bioanalytical methods [16,23,24,33,37,79–85]. Facilities in Hage's group can be used with both covalent and noncovalent immobilization methods to place proteins, drugs, nucleic acids or other agents on inorganic materials, organic polymers or carbohydrate-based materials (e.g., silica or glass, methacrylate or agarose). As one example, Hage's group has developed a method that can be used for the site-selective immobilization of antibodies to silica through their carbohydrate chains [79]. The same group has optimized techniques for immobilization of the proteins HSA, α_1-acid glycoprotein and high-density lipoprotein , and has recently reported a noncovalent entrapment method for protein and ligand immobilization that can be used with silica or other porous HPLC supports [21,23,24,33–35,37,80,82,83,85]. A variety of tools have been used in the creation and optimization of these immobilization methods. These tools have included protein assays, infrared spectroscopy, solid-state NMR spectroscopy and MS, as well as HPLC and CE [57,79–81,83–87].

Along with new methods for preparing stationary phases, additional work is being conducted in the Hage group for the creation of improved support materials for HPLC and related methods. A set of supports that are of current interest are monolithic materials [88,89]. Monolith columns consist of a continuous bed of a porous material that contains a stationary phase, but also allows flow of a mobile phase through the bed. Monolith columns are of great interest in HPLC because of their good mass transfer properties and low back pressures, allowing these materials to provide efficient separations at high-flow rates. The Hage group has been developing affinity monolith supports for use with antibodies, proteins and other binding agents [88,89]. These studies have included monoliths based on organic copolymers (e.g., glycidyl methacrylate and ethylene dimethacrylate) and silica monoliths (Figure 6). These materials have been used in applications that include ultrafast immunoextraction, HPAC assays for pharmaceutical agents, chiral separations and studies of drug–protein binding [16,23,24,35,49].

The Center for Nanohybrid Functional Materials has recently been created at UNL to further investigate and create novel materials for use in applications such as HPLC, biosensing and bioanalytical separations. This center consists of 15 faculty members from UNL, the University of Nebraska Medical Center, the University of Nebraska – Kearney, Creighton University (NE, USA) and Doane College (NE, USA). The goal of this center is to create, test and utilize new materials that consist of ordered nanostructural scaffolds that are functionalized or hybridized with chemical and biological recognition elements. These nanoscaffolds are being prepared with control of 3D morphology and with a variety of frameworks, one set of which includes an expanded set of supports for monolithic columns. As these scaffolds are developed they will be combined with binding agents such as antibodies or aptamers for use in flow-based biosensors or bioanalytical separations.

Executive summary

- Research efforts in the field of bioanalysis and separations at the Chemistry Department of University of Nebraska – Lincoln (UNL) include work with HPLC, CE, MS, NMR spectroscopy and several other techniques.
- One area of active research at UNL is in the development of novel separation methods based on high-performance affinity chromatography and affinity CE, such as the use of antibodies in HPLC systems to create chromatographic immunoassays or multidimensional separation methods.
- The use of separation systems such as high-performance affinity chromatography or affinity CE for the study of biological interactions and for chiral separations is another field of ongoing research at UNL. This work is of interest for the creation of new methods for the high-throughput screening of drug candidates or for predicting and modeling the behavior of drugs and proteins in the body.
- The techniques of LC–MS, GC–MS and NMR are being used to discover new natural products and to study their biosynthetic mechanisms. In addition, affinity separations are being used with MS and LC–MS to study and relate the effects of protein modifications with changes in protein function.
- Gas-phase ion mobility is being used for the gas-phase separation of isomeric biomolecules. This method is also being explored as a means for sorting different classes of biomolecules according to their masses, charges and collisional cross-sections.
- The combined use of structural, biological and NMR ligand-affinity screens with bioinformatics is being used to generate functional assignments for proteins. NMR spectroscopy is also being used for studies in metabolomics.
- Several efforts are underway at UNL regarding the creation of new supports and separation media for bioanalysis. Two examples are the generation of improved immobilization methods for use with biomolecules and the development of monolith materials for use in high-performance affinity separations. A new center aimed at the creation of nanohybrid functional materials has also been created at UNL.



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No writing assistance was utilized in the production of this manuscript.

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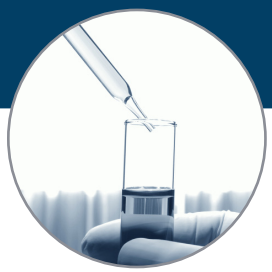

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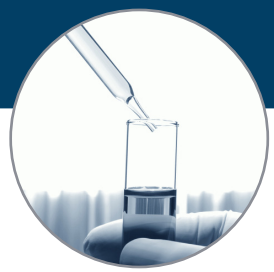

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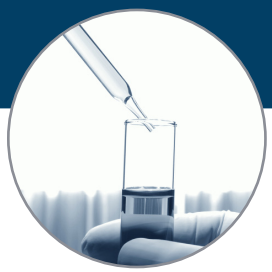

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