AFFINITY CHROMATOGRAPHY MASS SPECTROMETRY ASSAYS

AFFINITY CHROMATOGRAPHY MASS SPECTROMETRY ASSAYS FOR SMALL MOLECULE SCREENING

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LAY ABSTRACT

The discovery of new drug compounds is crucial for the treatment of diseases. Enzymes are proteins that turn a substrate into a product; and in diseases they can often malfunction, overproducing the product. Small molecule compounds can sometimes inhibit enzyme function and can be further developed into therapeutic drugs. This thesis describes a method for detecting small molecule inhibitors that bind to an enzyme that is immobilized in a small column. Once the small molecule is bound to the immobilized enzyme, it can be detected by either showing that enzyme function is inhibited or by removing the compound from the enzyme and identifying the compound by mass spectrometry. These methods can quickly identify compounds at extremely low levels from complex mixtures, such as natural product extracts.

ABSTRACT

Enzymes are implicated in many diseases including neurodegenerative, cancer, immune deficiency, and inflammatory disorders. There is a constant need to develop novel drug compounds that target enzymes in order to modulate their function, thus treating the disease state. These compounds are typically small molecules with affinity to the enzyme active site or an allosteric site. In order to discover novel compounds for treating disease, the interaction between an enzyme and a small molecule must first be identified and then characterized. With the target enzyme known, it is beneficial to screen libraries of compounds against the target. Immobilizing the enzyme allows for pre-concentration of ligands on the surface and therefore increased signal enhancement, as well as permitting multiple wash steps and enzyme reuse. Immobilized enzyme columns are optimal for coupling to a variety of detection devices by way of liquid chromatography, including absorbance or mass spectrometric detection. Immobilized enzyme reactors (IMERs) were generated and optimized for two target molecules, acetylcholinesterase (AChE) and adenosine deaminase (ADA), for rapid function-based screening of enzyme inhibitors in mixtures. The IMER mode is useful for increasing throughput and facilitating the identification of hit mixtures, but it is slow and tedious to manually deconvolute hit compounds from mixtures and the IMER method is not amenable to natural product extracts, which are good sources of structurally diverse compounds that are more likely to result in a hit compound. Bio-selective solid-phase extraction (BioSPE) is an orthogonal method of isolating and identifying enzyme inhibitors in a single step, and was used to easily deconvolute complex mixtures, rapidly

identifying to key compounds EHNA and MAC-0038732 out of mixtures using ADA columns. A data dependent acquisition MS method was developed and used to screen a set of fungal endophyte extracts, identifying two potentially novel inhibitors that were confirmed by IMER-MS/MS.

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LIST OF ABBREVIATIONS

AA	acetic acid
AChE	acetylcholinesterase
ADA	adenosine deaminase
APTES	aminopropyltriethoxysilane
ATCh	acetylthiocholine
BioSPE	bioselective solid phase extraction
CDC	competitive displacement chromatography
DDA	data dependent acquisition
DGS	diglycerylsilane
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine
EMS	enhanced mass spectrum
EPI	enhanced product ion
FAC	frontal affinity chromatography
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
HTS	high-throughput screening
IMER	immobilized enzyme reactor
K_D	dissociation constant
K_I	inhibition constant
K_M	Michaelis-Menten constant

LC	liquid chromatography
LIT	linear ion trap
MS	mass spectrometry
NMR	nuclear magnetic resonance spectroscopy
P/S	product-to-substrate ratio
UV-Vis	ultraviolet and visible spectroscopy

DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis embodies the research that Erica Forsberg has accomplished to develop continuous flow immobilized enzyme reactor screening and bio-selective solid phase extraction assays, both of which are novel methods for screening an enzyme target with potential inhibitors for the discovery of lead compounds for drug development.

In Chapter 2, James R.A. Green initially developed AChE columns while the author prepared the silica precursor, further optimized the column fabrication method and automated the screening assay moving from absorbance to MS detection. The author performed all data analysis and wrote the manuscript with Dr. John Brennan.

Chapter 3 contains research conducted solely by the author including optimization of ADA columns, automating the screening assay, developing data dependent MS methods for detecting inhibitors in mixtures and all data analysis. Dr. John Brennan and the author prepared the manuscript.

Emily Kapteyn and the author prepared columns for the research discussed in Chapter 4. Ashraf Ibrahim prepared the endophytic fungal extracts for the bioSPE screening assay and discussed screening results with the author. The author performed all data analysis and wrote the manuscript with Dr. John Brennan.

Dr. John D. Brennan supervised and made recommendations for all research in this document. Dr. Brian McCarry, Dr. Nathan Magarvey and Dr. Jose Moran-Mirabel also oversaw and made recommendations on this research.

PREFACE

This thesis has been prepared as a sandwich thesis with publications from the author as the major contributor. The introduction chapter contains sections of review articles from the following publications for which the author was the major contributor or was a co-author, where the author wrote the related sections.

Forsberg, E.M., Sicard, C., Brennan, J.D., **Solid-Phase Biological Assays for Drug Discovery**, Annual Reviews in Analytical Chemistry, **2014**, 7, 337-359.

Monton, M.R.N., Forsberg, E.M., Brennan, J.D., **Tailoring Sol-Gel Derived Silica Materials for Optical Biosensing**, Chemistry of Materials, 2012, 24(5), 796-811.

Lebert, J.M., Forsberg, E.M., Brennan, J.D., **Solid-phase assays for small molecule screening using sol-gel entrapped proteins**, Biochemistry and Cell Biology, **2008**, 86(2), 100-110.

Chapter 2 includes research on the development of AChE IMERs for screening mass encoded mixtures. The author conducted the AChE column fabrication optimization, mixture preparation and screening, as well as data analysis. James R.A. Green conducted initial column development and proof of principal of AChE columns. Dr. John Brennan and the author prepared the manuscript for publication.

Chapter 3 contains research on the development of ADA columns for use as IMERs to screen mass encoded mixtures and to perform bioSPE to isolate and identify inhibitors from the mixtures. The author optimized column fabrication methods and parameters for automated screening and mass spectrometric data dependent acquisition methods. The author conducted all data analysis and prepared the manuscript with Dr. John Brennan.

Chapter 4 is comprised of research on the limits of detection and complexity of the bioSPE assay and its application to screening highly complex natural product extracts versus ADA. The author and Emily Kapteyn prepared the ADA columns for mixture screening. The author prepared the compound mixtures for screening, tested the limits of detection and complexity of the assay and analyzed the data. Ashraf Ibrahim prepared the fungal endophyte extracts and assisted with data analysis for the metabolite extract screen.

Data obtained for this research is based on the need for developing novel screening technologies for the discovery of novel drug compounds. All work is original and groundbreaking for rapidly analyzing mixtures and/or natural product extracts for identifying and quantifying enzyme inhibitors.

The author would like to thank the supervisor, Dr. John D. Brennan for guidance and funding, as well as the National Science and Engineering Research Council and the Government of Ontario for scholarship funding. The author would also like to acknowledge the Biointerfaces Institute at McMaster University, particularly Dawn White and Medhi Keramane, for assistance with research projects, Jenny Wang of the High-Throughput Screening Facility at the Centre for Microbial Chemical Biology for assistance in selecting and mixing compound libraries, and Dr. Leslie Silva for assistance with editing.

CHAPTER 1. INTRODUCTION

Overview

The discovery of novel enzyme inhibitors plays an important role in the development of new therapeutics. There is a constant need to find novel ways of detecting protein-ligand interactions, particularly from sources that contain structurally diverse bioactive molecules. This thesis describes the development of immobilized enzyme columns for the screening and discovery of inhibitors from complex mixtures. Herein, the importance for an immobilized protein platform is described, with a discussion of clinically relevant drug targets and their ability to be immobilized. Protein entrapment by the sol–gel process has been used extensively to immobilize proteins, allowing for the study of interactions of small molecules with an immobilized target, and this method is also discussed in detail as was the primary method used for developing immobilized protein columns. A selection of relevant methods for using bioaffinity columns to screen for enzyme inhibitors is introduced prior to their discussion in greater detail within the following chapters. The sources of small molecules for screening are then considered, specifically discussing synthetic chemical libraries, directed bioactive libraries and the highly diverse natural product extracts, including fungal endophytes that are screened in Chapter 4.

Solid-Phase Biological Assays

Biological assays are ubiquitous in a wide range of areas, from clinical diagnostics to environmental analysis to food and beverage testing, and include such techniques as enzyme assays, immunoassays, and assays using DNA hybridization, often coupled with the polymerase chain reaction.[1] While a large number of such assays have been reported for biosensor and other biodetection applications, far less emphasis has been placed on solid-phase biological assays (SPBA) in the area of drug discovery, particularly for small molecule screening to identify drug leads.[2,3] This is slowly changing, as there are several advantages to moving small molecule screening assays to the solid phase by immobilizing one or more assay components onto a solid support such as plastic, metal or glass. For example, solid phase assays make it possible to utilize a number of new assay formats (microarrays, columns, microfluidic chips, etc.), use novel surface-dependent signaling methods (surface plasmon resonance, total internal reflection fluorescence, etc.), and provide a facile means of performing flow injection or multi-step reactions that require washing steps or addition of reagents to generate signals.[2,4,5] In some cases, SPBA can provide unique advantages such as multiplexed screening via microarrays,[6] facile extraction of bioactive compounds from mixtures using chromatographic bioextraction methods,[7] or can provide a platform to increase selectivity by introducing marker compounds that allow competitive assays for site specific targeting.[8] Such assays can also increase assay throughput since many assays can be designed to utilize small molecule mixtures as inputs, and can be automated by integrating them with advanced liquid handlers or autosamplers.[9]

To create an SPBA, one must have a biorecognition element (e.g. a biomolecule that is the drug target), a method to immobilize it on or within a suitable surface, and a mechanism to transduce the binding event between the biomolecule and bioactive compound into a measurable signal (Figure 1.1). Additional steps, such as purification or preconcentration,[10] may also be required and in many cases a method to amplify the initial signal[11] may be required to obtain suitable detection limits for compounds that may be present at low levels in complex mixtures.

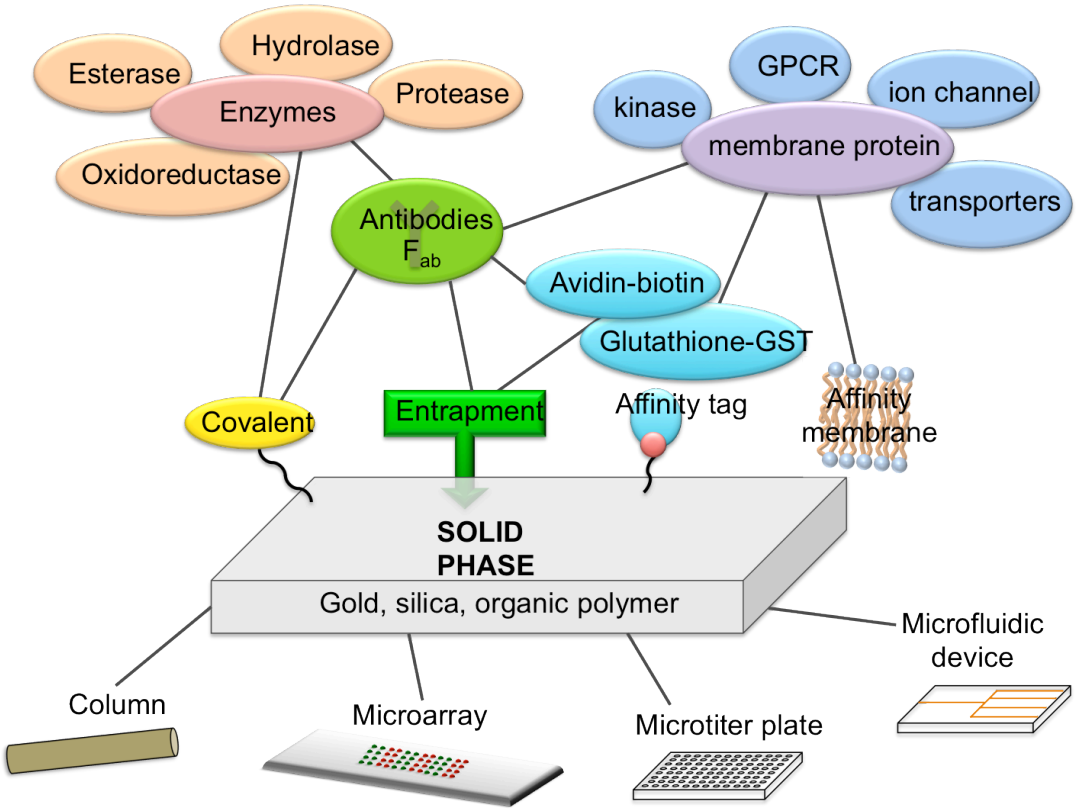

Figure 1.1. A general schematic for solid-phase biological assays is illustrated above and are typically comprised of a biorecognition element or biomolecule that is immobilized on or in a solid surface, which must then be coupled to a signal transduction method to detect the presence of a binding event. Reproduced with permission © 2014 Annual Reviews.

While the immobilization of biomolecules, and particularly soluble proteins, on a solid-phase has been utilized for decades as a method to detect biological interactions, there are still many challenges in the field. Reasons for this include a lack of biomolecule stability once immobilized,[3,9] difficulty with immobilizing labile biomolecules such as membrane receptors,[3,12] poor quantitative responses for certain assay formats, which makes it hard to rank "hits" in order of potency, and potential issues with false positives and negatives owing to non-specific binding to the solid support.[13]

Enzymes as Biological Targets for Drug Discovery

Enzymes are biological catalysts that accelerate the rate of biological reactions by converting substrates to products. Typically, small molecules modulate enzyme activity by directly binding to the active site (competitively or non-competitively), binding at an allosteric site, or blocking binding of essential co-factors. The use of enzymes for solid-phase assays in the drug-discovery arena dates back over 30 years, where Kawauchi and co-workers used an immobilized enzyme electrode for inhibitor screening against β-D-glucose oxidase.[14] Since this time, a wide range of soluble enzymes, including various oxidoreductases,[15] proteases,[16] esterases,[17] kinases,[18] and dehydrogenases,[19] to name a few, have been immobilized or entrapped in a wide variety of materials and used for screening of inhibitors.

Most enzymes are soluble (with the exception of lipases and receptor tyrosine kinases[20]), and thus the polarity of the solid-phase must be designed to be relatively hydrophilic in order to optimize the native conformation. However, some classes of

clinically relevant soluble enzymes are quite labile and thus challenging to immobilize, such as kinases. Other key enzymes for drug discovery in the solid phase are proteases and transcriptases, both of which are important in development of HIV treatments and in cancer treatments.[21,22] The caspases are key enzymes involved in apoptosis, and thus in regulation of cancer.[23] These enzymes act on either DNA or protein-based substrates, and regulate processes related to infection and cell cycle. Neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) are often associated with membrane receptor targets, yet small molecule inhibitors targeting soluble enzymes, such as the divalent metal transport protein, acetylcholinesterase and monoamine oxidase, show efficacy in the treatment of such cognitive disorders.[24] Neurodegenerative diseases are also amongst a large group of diseases that invoke an inflammatory response involving the catabolic enzyme adenosine deaminase, which has recently come to attention as a potential drug target for its role in the regulation of immune functions.[25] As such, both AChE and ADA are important targets for drug discovery.

Immobilization of Soluble Proteins

There are several different methods by which soluble biomolecules can be immobilized. The most common methods include physical adsorption, covalent binding, affinity based techniques, and encapsulation within porous polymeric or composite organic-inorganic materials. Physical adsorption involves the use of weak interactions such as hydrogen bonding, hydrophobic interactions, or electrostatic interactions between the biomolecule and the surface. While physical adsorption is relatively easy to perform,

there is little control over biomolecule orientation, biomolecules may undergo undesirable conformational changes upon adsorption, and minor alterations in solvent conditions, pH or temperature can lead to desorption of the biomolecule.[26] Such issues can be partially overcome by entrapping the protein in films prepared by the layer-by-layer method, which involves the formation of separate films of opposing charge sandwiching the biorecognition element. This approach gives good control over film thickness and molecular architecture but must be tuned in order to preserve protein orientation and accessibility.[27]

Covalent immobilization methods prevent leaching or desorption of the biomolecule as they involve a crosslinking reagent that conjugates a reactive functional group on the surface of the folded protein, such as cysteines (thiols) or lysines (amines), with a suitably activated surface including aldehyde, carboxylic acid, amine or hydroxyl modifications. Common crosslinkers include glutaraldehyde, carbonyldiimidizole, N-hydroxysuccinimidyl ester, maleimides, epoxides and photo-reactive species.[28] A limitation of this method is that most biomolecules can contain multiple cysteine or lysine residues, making it difficult to control the orientation of the biomolecule leading to a loss in active site accessibility. Using a protein-to-crosslinker ratio $\geq$ 1:1 is key to ensuring a single site of protein attachment to the surface.[29]

Affinity-based immobilization techniques make use of natural biomolecular binding interactions to specifically tether the biomolecule to a suitably modified substrate. Examples include biotin-avidin interactions, aptamer capture, antibody capture, hexahistidine tags to bind to Ni^{2+}-nitrilo triacetic acid derivatized surfaces and

glutathione-S-transferase tags to bind to glutathione modified surfaces.[26] These affinity methods often allow for some control over orientation but require recombinant proteins and typically are not amenable to intrinsic or extrinsic membrane proteins or highly hydrophobic proteins such as lipases, which tend to aggregate upon immobilization.

A final method is entrapment into polymeric materials, which can include a variety of organic polymers (carboxymethylated dextran, agarose, chitosan, alginate, polypyrrole) acrylate-based hydrogels and inorganic or hybrid materials, most often obtained by the sol-gel method using alkoxysilanes and related species (i.e., diglyceryl silane, sodium silicate, silicic acid). Such materials are generally formed by condensation of monomers around a biomolecule, and can be cast as films, monoliths or columns, printed as microarrays or as coatings on surfaces, or formed as biomolecule-doped particles.[30] The advantages of such an approach to immobilization is the versatility of surface chemistries, ability to control pore sizes to produce a size exclusion barrier (which can prevent degradation of entrapped species by proteases or nucleases), tunable surface chemistry and the ability to add dopants to provide biomolecule stability or to allow signal development (i.e. via optical dyes or metal particles). However, optimizing such materials can be a time and labor intensive process, although recent high-throughput optimization processes have helped accelerate the development of such materials.[31]

Sol-Gel Processing and Materials

The sol-gel process is a room temperature technique for synthesizing porous, glass-like materials and ceramics[32,33] that has found applications across many disciplines, such as optics, electronics, nanotechnology, medicine, biology, chemistry, materials and

separation sciences. At the interface of biology, chemistry and materials science, the immobilization of biomolecules via entrapment (or encapsulation) within a sol-gel matrix, and to a more limited extent, by attachment onto sol-gel derived surfaces, have led to some of the most interesting and important applications of sol-gel derived materials, and have paved the way for their use in the design and fabrication of bioanalytical devices for rapid detection of analytes in a variety of matrixes. In the two decades following the publication in 1990 of Braun *et al.*'s pioneering work describing the successful entrapment of the enzyme alkaline phosphatase (AlP) in alkoxy-silane-derived glass,[34] there has been tremendous progress in the area of bioencapsulation in sol–gel-derived materials, buoyed in part by the development of biocompatible precursors and processing conditions, and the availability of different techniques for probing the behaviour of bioencapsulates. This has enabled immobilization of a wider range of biologically active elements, including highly sensitive and fragile species, such as living cells, organelles, kinases and membrane-bound receptors,[35,36] and has opened the field to new areas of application including identification and characterization of protein-ligand interactions. A number of excellent reviews covering various aspects of sol–gel materials and their uses have been published.[33,35-50]

Sol-gel processing involves the formation of metal or semi-metal oxides via aqueous processing of hydrolytically labile precursors as shown in Figure 1.2.[51-53] Although bioencapsulation in other oxides such as TiO_2,[54-56] Al_2O_3,[57-59] and ZrO_2[60,61] have been reported, SiO_2 is the most well studied by far.[62] Traditionally, an alkoxide precusor (e.g., tetramethylorthosilicate (TMOS) or tetraethylorthosilicate (TEOS) is

mixed with water and a mutual solvent (i.e., alcohol).[63] The hydrolysis reaction forms silanols, silanols then condense together to form siloxanes, and finally, through polycondensation, silanols react with siloxanes to form a rigid, porous network of interconnected silica after drying and aging under ambient conditions.[41,63] The biomolecule is usually added to the sol after partial hydrolysis of the precursor, and as the degree of cross-linking from polycondensation increases, the sol becomes more viscous and eventually gels, entrapping the biomolecule within its pores.[41,64]

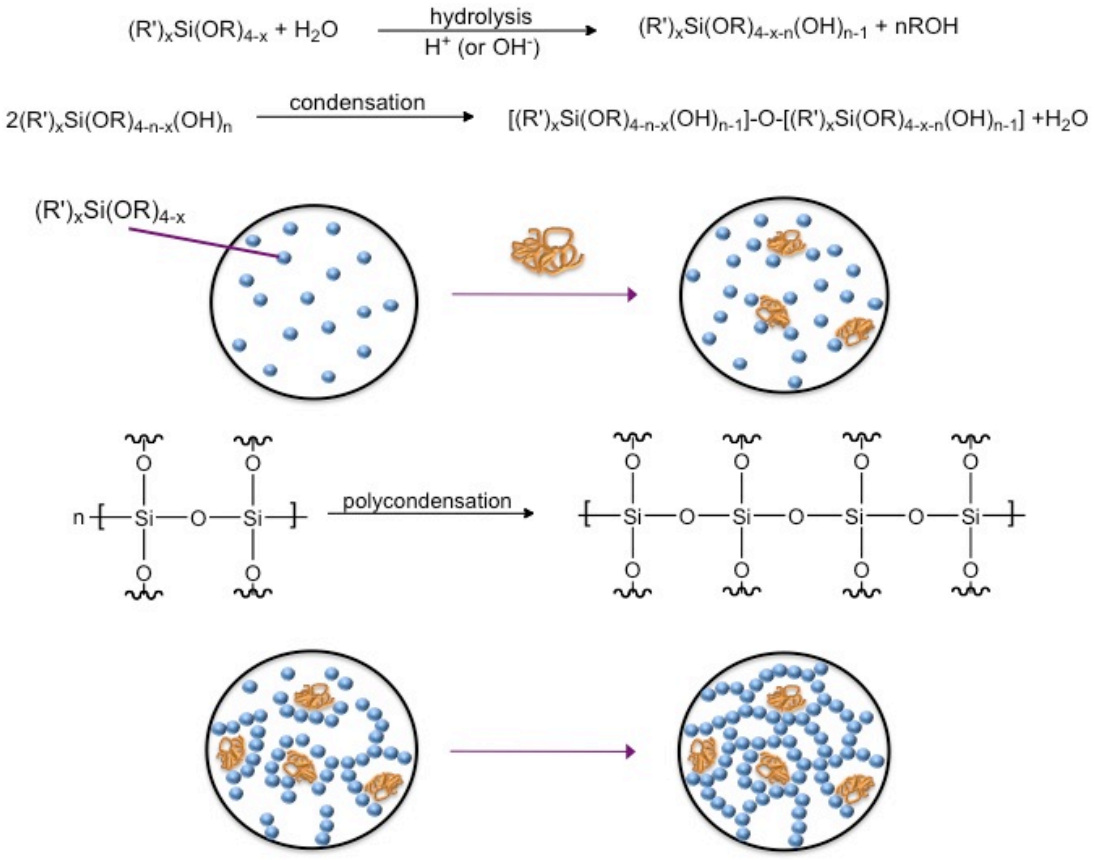

Figure 1.2. The formation of sol-gel derived silica glass with protein entrapment is performed by hydrolysis of labile silica precursors usually in water or a mutual solvent. Upon condensation of the sol, biomolecules may be added to perform encapsulation. During polycondensation, the material gels to form a solid three dimensionally cross-linked porous structure.

Sol–Gel Processing for Bioencapsulation

Several properties of sol-gel derived materials, most notably, those that are silica-based, render them particularly compatible with many different biological assay formats, from biosensors to bioaffinity chromatography columns to microarrays. As a solid-phase support material, they are mechanically robust, chemically inert and resistant to thermal, photochemical and biodegradation.[40,46,65,66] The silica framework grows around the guest biomolecules resulting in an increase of the dopant size upper limit.[66,67] The interpenetrating networks of silica effectively serve to "cage" the biomolecules, preventing them from leaching, but preclude macromolecular exchange and thus protein-protein interactions cannot be detected.[41,47,65]

The ability of an entrapped biomolecule to retain its functionality and maintain its stability, and eventually, the analytical performance of the device in which it is employed, are largely determined by the nature of the local microenvironment that it encounters within the pore. For instance, electrostatic interactions between the silica wall and a guest protein can influence the latter's rotational freedom – cationic proteins have been shown to have hindered dynamics, whereas neutral or anionic proteins are known to have less restricted motions.[41,68-71] Similarly, these interactions can affect the accessibility of the entrapped biomolecule to external analytes, even though the pore sizes are adequate for unrestricted transport of small molecules including buffer ions, substrates and products of reactions in and out of the porous structure.[35,41,65,72] Thus, considerable work has been focused on designing precursors and additives, and devising processing protocols for the purpose of controlling the properties of the silica matrix to make it a hospitable host for

the dopant molecule, and reducing the effects of the changes in the material, such as shrinkage, drying and cracking, that can occur with its continued evolution over time.[41,73]

Provided the microenvironment of the material has been properly tuned, biomolecules are able to retain many of the characteristics in the liquid state:[64] enzymes and DNAzymes remain catalytically active, antibodies and aptamers maintain their substrate binding affinities, and cells remain viable.[74] Multiple species can also be co-entrapped, allowing the use of coupled reactions.[75-78] In many cases, the entrapped biomolecules remain in their functional state over longer periods compared to their free forms, have increased resistance to denaturation,[34,79,80] or may even be reusable;[41] they can be easily cast in a variety of geometric configurations, such as monoliths, powders, thin film, fibres, arrays, or other more esoteric structures, thus lending flexibility to assay platform design.[41,46,66] They can also be easily miniaturized or attached to other materials.[44] Finally, the matrix provides a steric barrier protecting the entrapped biological element from potentially deactivating components of the sample (e.g., proteases for proteins, nucleases for aptamers),[81] just as it also protects the sample from direct exposure to the biomolecule[66] (e.g, for *in vivo* applications).

While sol–gel chemistry is intrinsically mild, conventional processing techniques and traditional precursors are not naturally suited to biologicals because they generally require extremes of pH and high concentrations of alcohol.[64] Initial efforts aimed at limiting exposure of labile biomolecules to potentially denaturing conditions included decoupling of the relatively harsh hydrolysis step from condensation by adding the silica sol to a buffered solution of the biomolecule,[82] and minimizing the amount of alcohol in

the system, by utilizing the alcohol released as a hydrolytic byproduct to homogenize water and alkoxide,[83] evaporating the alcohol under vacuum to fully hydrolyze the solution prior to addition of biomolecule,[84] or merging alcohol removal with the use of very high molar ratios of water to alkoxysilicate (~25:1 – 50:1) so that gelation occurs almost entirely in water.[85]

Processing can also be performed using a completely alcohol-free route: Bhatia et al.[42] adopted Dickey's original technique of using silicic acid, $Si(OH)_4$, using sodium silicate as the aqueous precursor,[86] but modified it by treating the silicate with an ion exchange resin to simultaneously remove sodium ions and to lower the pH of the sol, followed by gelation at neutral pH by addition of a suitable buffer containing the biomolecules. In this way, the biological activities of the encapsulated enzymes horseradish peroxidase (HRP) and glucose-6-phosphate dehydrogenase could be retained. This technique is still widely employed[87-89] as it affords some degree of control, albeit limited, over hydrolysis and condensation rates for tailoring the properties of the resulting gel. Polyol esters of silicates and siloxanes, particularly those derived from glycerol, such as poly(glyceryl silicate) (PGS)[90] and diglycerylsilane (DGS)[91,92] have also been demonstrated to retain the activities of bioencapsulates at levels approaching those of free biologicals. Because these precursors are highly water soluble, they allow high doping levels and processing to be performed at physiologic pH without the need for pH adjustment. Importantly, they release the non-volatile, osmoprotective byproduct glycerol during hydrolysis, which helps retain protein activity and also reduces shrinkage of the matrix during aging as the byproduct is not volatile.[36,90]

Properties of Entrapped Biomolecules

Physical entrapment in a sol–gel derived matrix confers a protective effect on the entrapped biomolecule,[66-68] since spatial restrictions limit the conformational changes (unfolding, rotation) that a biomolecule can undergo,[35,41] and reduce the likelihood of intermolecular associations (aggregation).[64,93] However, the confining pore causes the biomolecule to be subjected to conditions that can be drastically different from its native environment, and may depend greatly on the nature of the biomolecule. These conditions, taken together with the reduced degrees of freedom experienced by the biomolecule, can change its biological and biophysical properties. Thus, reports comparing the measured activities of bioencapsulates to their free forms can range widely, from a meager 1%,[94] a modest 30%,[34] a high 98%,[90] to an impressive 4000%,[80] although the discrepancies can be due as much to the nature of the biomolecules themselves as the effect of entrapment.

There is a vast field of literature looking at the behaviour of biomolecules entrapped in sol-gel derived materials.[69,71,72,90,93,95-110] These studies, which look at protein conformation, rotational and translational dynamics, kinetics, and interactions with the pore surface, have provided useful insights into the fundamental factors that may affect biomolecules during and after entrapment.[36] This makes it possible to rationally design materials in order to better sustain biological function over extended periods of time, while maintaining fabrication requirements and eliciting optimal analytical performance. The properties of the silica matrix that are usually optimized for a specific biomolecule include polarity, surface charge profiles, pore morphology and mechanical stability. The complex interplay among these properties means that controlling for one

inevitably affects the others. It is widely regarded that a change in a processing step or the presence of an additional component in the matrix can modify two or more properties simultaneously, and the apparent change in the structure of the material, or in the activity or stability of the encapsulated biomolecule, arise from the additive or synergistic effects of these modifications (e.g, polymers, ionic strength and pH alter hydrolysis rates, pore morphology, and protein–silica interactions).[97,111]

For optimum performance in bioaffinity chromatography applications, the bio-doped silica matrix should ideally have a highly porous structure, with large surface areas for enhanced reactivity and improved recognition properties, and wide, open pores for rapid mass transport.[46] Similar to silica–analyte interactions, the pore size is directly correlated with reaction kinetics. In the entrapped format, the kinetics of reactions involving biomolecules are known to be slower than in solution, with the catalytic and binding rates often reduced,[34,73,112-117] and the reduction rates considered to be due to mass transfer limitations and partitioning effects.[41,66,117] For enzymes, K_M values typically increase, and V_{max} values decrease.[117] Because the pores are not straight, analytes diffuse more slowly as they need to cover longer distances,[118] as well as overcome the increased viscosity of entrapped solvents.[41] One way to aid in analyte accessibility is to use pressure driven flow through a monolithic column. A restriction of this format is the need for a bimodal pore distribution within the silica matrix. Mesopores are in the range of 2 to 50 nm and are required to encapsulate the biomolecule. Macropores are 50 to 1000 nm and necessary to alleviate backpressure when flow is applied. Polymers such as polyvinyl alcohol[108,119,120], polyethylene glycol (PEG),[107,119,121]

and hydroxyethyl carboxymethyl cellulose[122] have been commonly used as pore-formers, with the resulting materials exhibiting micron-scale pores along with nanometer sized mesopores, and showing minimal shrinkage during drying because of the polymer "pore filling" effect.[123,124] One of the most effective and well characterized methods of fabricating biocompatible sol-gel derived silica columns is using PEG as the porogen, resulting in the desired bimodal pore distribution and is easily amenable to liquid chromatography.[125-127]

Fabrication of Sol-Gel Derived Columns

The fabrication of bioaffinity columns involves forming a protein-doped sol and then infusing it into a capillary column prior to gelation to produce a monolithic bed that contains entrapped proteins. Specific pore morphology (i.e., size of pores and proportion of meso and macropores) can be controlled by varying pH, PEG molecular weight and percentage of PEG present in the sol. In optimal cases the porosity is such that pressure-driven flow in the low μL/min range is possible while still retaining over 80% of the entrapped biomolecules.[128] Columns are typically prepared using a mixture of the protein-compatible silica precursor diglycerylsilane (DGS),[129] PEG (MW 10 kDa), which controls pore size distribution, aminopropyltriethoxysilane (APTES), which provides cationic sites that counterbalance the anionic charge of the silica to reduce non-selective interactions, and a buffered solution of the protein of interest to provide bioaffinity sites within the column. The resulting sol mixture is loaded into fused silica capillaries (150 - 250 μm i.d.), whereupon spinodal decomposition of the PEG-containing sol occurs, followed by gelation of the silica.[126,127,130] The evolution of glycerol as a byproduct of

DGS hydrolysis maintains the entrapped proteins in an active state during column aging, yet is removed from the column during the initial column flushing step owing to its small size relative to the protein, preventing the elution of glycerol into the detector. Although conditions for protein entrapment vary depending on the biomolecule, typical pH values for macroporous bioaffinity columns can range from 7.0 to 7.4 while PEG content can range from 2 – 8 wt%.

Regardless of the application being used for the bioaffinity column, there is a need to prepare protein-loaded columns with high activity and high binding site density. The predominant method for fabrication of columns containing soluble proteins has been based on covalent or affinity coupling of such proteins to silica beads. However, covalent coupling of proteins to beads has several limitations, including loss of activity upon coupling (due to poor control over protein orientation and conformation),[28] low surface area, potentially high backpressure which may alter K_d values,[131] difficulty in the loading of beads into narrow bore columns, difficulty in miniaturizing to very narrow columns (<50 μm i.d.) to allow low volume screening and poor versatility. On the other hand, covalent coupling can also be applied to monolithic silica columns prepared in the absence of a biomolecule, which allows for better control of the sol–gel material formation while still resulting in high surface area materials that can still be miniaturized. Column fabrication byproducts, such as PEG and glycerol, must be thoroughly removed prior to biomolecule coupling when more sensitive mass spectrometric applications are desired.

Application of Bioaffinity Columns to Screening

The majority of screening methods currently in use are designed for screening large numbers of discrete compounds. However, an emerging method to increase throughput is the assaying of mixtures of compounds, either coupled to or followed by a deconvolution step to identify specific inhibitory compounds within bioactive mixtures. Among the various affinity methods used for screening mixtures[132,133] are a series of solid-phase assays that utilize proteins immobilized into columns to allow various affinity chromatography-based methods to be employed for mixture screening. One of the most prevalent modes of column-based screening is frontal affinity chromatography (FAC),[134-137] where mixtures are continuously eluted through protein doped columns, either alone or with a known inhibitor that acts as an indicator ligand in competitive binding assays. In the absence of an indicator ligand the compounds in a mixture will elute in the inverse order of their affinity for the immobilized protein. When an indicator compound is present, the indicator will show a transient over-concentration, or "roll-up", when a ligand that binds to the same site as the indicator is present in the mixture. Using such methods makes it possible to screen mixtures against a range of proteins, including enzymes, regulatory proteins and membrane-associated receptors.[138-140]

FAC-MS/MS Using Soluble Proteins

Frontal affinity chromatography coupled with mass spectrometry has been a powerful tool to identify and characterize soluble protein–ligand interactions. Initial studies in our research group used sol-gel derived bioaffinity columns for screening simple mixtures against immobilized dihydrofolate reductase (DHFR) using FAC interfaced to ESI-

MS/MS.[130] DHFR catalyzes the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of dihydrofolate (DHF) to tetrahydrofolate, which is then used as a co-factor in the biosynthesis of thymidylate, purines and several amino acids.[141] DHFR is an essential enzyme in the cell and is the target for antifolate drugs.[142] The FAC-MS/MS assay involves continuous infusion of a compound that allows for equilibration of the ligand between the free and bound states, where the precise concentration of free ligand is known. In this case, the breakthrough time of the compound will correspond to the affinity of the ligand for the immobilized biomolecule – ligands with higher affinity will break through later. This study showed that known inhibitors were retained in the bioaffinity column in a manner that was dependent on their dissociation constant (K_d) values. In addition, by monitoring the breakthrough volume as a function of analyte concentration, it was possible to determine both the binding site density (B_t in picomoles), and ligand dissociation constant (K_d, in μM) using the following equation:[138]

$$V = V_0 + \frac{B_t}{[A] + K_d} \quad (1)$$

where V_0 is the void volume (μL), V is the retention volume (μL) and [A] is the analyte concentration (μM). The ligand binding study showed that 10 pmol of active protein was present on the column (25% of the initially loaded protein) and that the ligand binding constants agreed with those obtained using standard solution assays. This demonstrated the ability to detect inhibitors present in compound mixtures using small amounts of immobilized protein.

Immobilized Enzyme Reactors

FAC-MS studies are ideal when the compounds of interest are known and characterized for analysis by multiple reaction monitoring-mass spectrometry (MRM-MS). However, when performing a mixture screen, a large number of compounds may be present and all cannot be monitored simultaneously using MRM analysis. In addition, unless a competitive ligand with a known site of action is used, the retention of a ligand provides no information on the site or mode of binding. Thus, it is useful to have a method that is based on changes in the activity of an immobilized biomolecules (i.e., substrate turnover by an enzyme) to garner information on the specific mode of inhibitor action. One method to achieve this goal is to use the immobilized enzyme reactor format directly interfaced with tandem mass spectrometry (IMER-MS/MS).[143] Several research groups have reported on the use of IMER technology for screening of compounds, though most of these either did not utilize MS for detection or utilized a second chromatographic step to separate substrate and product prior to MS analysis.[144,145] IMER-MS methods have potential advantages over the more conventional use of MS to monitor solution based enzyme reactions in that the enzyme can be reused, saving on reagent costs, and can also be used for subsequent affinity assays to deconvolute bioactive mixtures using FAC or bio-selective solid-phase extraction (bioSPE) methods.[140,144,146]

Using sol-gel derived columns containing the enzyme adenosine deaminase (ADA), our group developed an automated, continuous flow IMER-MS/MS method that was amenable to mixture screening, and demonstrated that the MS/MS method could be used to quantify both product and substrate species, removing the need for a

chromatographic separation step prior to MS analysis.[143] The IMER-MS/MS method involves continuous infusion of a substrate solution through an enzyme-doped column, where it is partially converted to product molecules. Using MRM, ions specific to the substrate and product are monitored independently to obtain a product-to-substrate (P/S) ratio. Since the sum of S+P is a known constant within the system, the P/S ratio can easily and accurately be used to determine the concentration of product eluting from the column. When mixtures of compounds are introduced into the substrate stream (note that these mixtures also contain a substrate concentration identical to that in the main flow stream to avoid dilution), the P/S ratio will remain constant if no inhibitor is present, but will be altered in favour of substrate if an inhibitor is present. In the case of ADA-doped columns, the conversion of adenosine to inosine was monitored and it was shown that mixtures containing the known inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) resulted in a significant decrease in the P/S signal ratio, indicative of enzyme inhibition. Use of an autosampler in the nanoLC-MS system allowed for rapid screening of several mixtures using a single column.

Potential advantages of the IMER-MS/MS method include the ability to monitor a wide variety of substrates and products with no need for labels, the ability to automate the assay and to apply it to mixtures, and the low volumes of reagents used per screen ($\leq$20 μL per mixture). However, the method requires enzymes that produce relatively rapid turnover of substrate, as the contact time for substrates on the column is on the order of 1 min. While longer contact time can be achieved using longer columns or slower flow rates, these approaches increase assay time. In such cases off-line enzyme

assays may be more appropriate, followed by automated injection into the MS/MS system to observe changes in P/S signal ratios.

Bio-Selective Solid-Phase Extraction

Another mode of column operation was also demonstrated in Hodgson's FAC study of DHFR, wherein the ligands in the mixture were pre-equilibrated with the protein on the column and then dissociated from the column using a wash with a solution containing high levels of the substrate, folic acid. This mode of operation, which we term bio-selective solid-phase extraction (bioSPE) provides a means to pre-concentrate high affinity ligands on the column, which then elute as a large peak upon introduction of the substrate, as shown in Figure 1.3.[130] This method has significant potential for screening of complex mixtures, since non-binding ligands will be removed immediately from the column and will not show a transient overconcentration, while higher affinity ligands will show large transient over-concentrations, making identification of such compounds straightforward. In addition, the use of different substrates that bind to different sites may provide information on sites and modes of inhibition, while washing with eluents such as acetic acid provides a means to identify allosteric inhibitors.

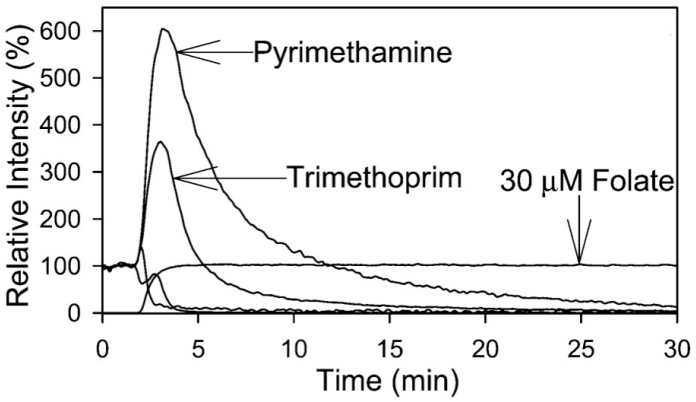

Figure 1.3. BioSPE of pyrimethamine and trimethoprim, simultaneously extracted from dihydrofolate reductase columns using 30 μM folate as a competitive displacer. Image is used with permission from ACS Publishing©.

Compound Screening Libraries

There is often a connection between enzyme inhibition and treatment of a disease state. For example, there is a strong association between inhibition of the enzyme acetylcholinesterase (AChE) and treatment of symptoms of Alzheimer's disease (AD).[147] Discovering novel enzyme inhibitors can be a long and tedious process, which can be aided by selecting a diverse compound screening library. Sources of small molecules to screen can be from synthetic chemical libraries, natural product sources or a combination of both.[148] Using known bioactive libraries is another way to direct the screen towards finding hits; they are already characterized and likely already approved for clinical use, which makes finding novel use for them as inhibitors in enzymatic systems desirable. By using known bioactive chemical libraries in the development of IMER and bioSPE, the screening assays can be improved to handle solvents such as DMSO, in which bioactive compound libraries are typically stored.

Alternatively, there is also a benefit in removing DMSO and focusing the screen on aqueous compound libraries. This biases the screen towards aqueous compounds that are often useful as lead compounds for the development of orally administered drugs.[149] Additionally, these screening platforms are require low micromolar concentrations, where most bioactive compounds are soluble. The addition of a low percentage of organic solvent, such as methanol, is also an option for aqueous-based screening assays since the immobilized enzymes tend to have greater tolerance to harsh conditions without adversely affecting their binding kinetics.[150] Although using known bioactive libraries is useful for assay development, it is desirable for these platforms to be amenable to natural product extracts that tend to contain structurally diverse secondary metabolites. This becomes challenging as mixture complexity can be high, concentrations tend to be unknown and structures are often uncharacterized.

Secondary Metabolites from Natural Product Extracts

Historically, the use of natural products (NPs) for drug discovery has been the best approach to treat illness or symptoms of illness.[151] Even in the past 25 years, two thirds of the pharmaceuticals on the market have been derived from NP sources.[152] Recently, there have been drawbacks to using present day HTS methods for drug discovery from NP extracts that tend to be aimed at synthetic compound libraries that can be more easily controlled for concentration and purity. The ability to reproducibly isolate metabolites from a complex matrix is often difficult owing to natural variability in successive metabolite extractions. It can also be difficult to elucidate highly complex structures, however, recent advances in NMR and MS techniques have shown promise in

successfully interpreting such structures.[152] Once a structure has been elucidated, it can be particularly cumbersome to synthesize the all too often convoluted structures of secondary metabolites.[153] However, NPs are still an excellent source for diverse secondary metabolite structures as they tend to exhibit large amounts of pharmacophores and stereochemistr,[153] and lipophilicity,[149] which play key roles in bioactivity.

Bioassay-guided fractionation and isolation is the principal method of assessing the bioactivity of secondary metabolites. This is where an extract is tested for bioactivity, and then separated into fractionated parts to be tested again to determine the bioactive fraction. This process is repeated until a purified fraction containing one compound with bioactivity is isolated. In many cases, over the course of this process, the functional component becomes too dilute over subsequent purifications to show bioactivity and the lead compound is lost. This problem may be addressed by applying bioSPE to natural product extracts, which is capable of preconcentrating the analyte and removing the inactive compounds in one simple assay. The paradigm of high-throughput screening does not easily lend itself to screen secondary metabolite extracts, yet that does not mean they should not be explored, but perhaps new technologies should be developed that better suits the source of the bioactive compounds.

Endophytic Fungi

There are many sources of secondary metabolites – plant, microorganisms (bacteria or fungi), animals – and many of them have not been well explored. Some of the most characterized sources of secondary metabolites from microorganisms are from the bacteria actinomycetes. Fungi are now also recognized as an excellent source of

structurally diverse bioactive molecules, yet the role of most fungal metabolites is not well understood. Endophytic fungi are no exception; there is a plethora of species that produce an abundant source of bioactive compounds. Some are toxic, such as alkaloidic mycotoxins commonly produced from *Clavicipitaceae* endophytes, which work symbiotically to protect their hosts. Non-*Clavicipitaceae* endophytes are not as well understood, but also capable of reducing stresses on plants by producing secondary metabolites. These compounds have also found great potential as therapeutics. Placitaxel is a well-known anti-cancer drug that is derived from the endophytic fungus *Taxomyces andreanae* and is just one example of the potential that lies in this unique source.[154]

Thesis Goals

Exploring the diverse source of secondary metabolites is useful in the search of novel therapeutics. Bioassay-guided fractionation and isolation is a cumbersome process that does not always lead to identifying the bioactive component due to the diluting effect of subsequent purification steps while commonplace HTS methods are not readily compatible with complex extracts. There remains a need to increase the screening rate of synthetic mixtures and NP extracts and improve assay sensitivity. This thesis explores the potential of immobilized enzyme columns, using either IMER or bioSPE based screening methods, to isolate, identify and confirm activity of inhibitors from complex mixtures. The goal of this research was to fully develop sol–gel derived bioaffinity columns containing immobilized enzymes, using AChE and ADA as examples, and apply them to highly complex bioactive samples. Chapter 2 describes the development of a continuous flow IMER-MS/MS platform used to screen a bioactive mixture compound

library against immobilized AChE. This work explores the limits of mixture complexity for the IMER method, evaluated optimal solvent conditions, and demonstrates the application of the IMER screening method for identifying bioactive compounds in mixtures that inhibit enzyme activity, as well as a method to deconvolute mixtures using IMER to identify the inhibitor. Chapter 3 describes the development of a new assay method, biologically selective solid-phase extraction, that provides a complimentary method to IMER and allows for more rapid deconvolution of bioactive mixtures. Columns with immobilized adenosine deaminase were prepared and initially used to screen mixtures by IMER-MS/MS, illustrating that this technology is compatible other enzymatic systems. BioSPE was then evaluated as a tool to identify bioactives in the hit mixtures, and was optimized to allow isolation and identification of inhibitors quickly using both mass encoded mixtures and a newly developed data dependent MS method. Chapter 4 evaluates the potential of the combined IMER/bioSPE screening method to screen more complex mixtures, including natural product extracts. In this chapter, the detection limits of bioSPE were assessed in a range of synthetic mixtures to determine the effects of mixture complexity on assay performance. Finally, bioSPE was applied to a series of fungal endophyte extracts. Two novel bioactive compounds were isolated to give preliminary structural information using data dependent MS and confirmation of ADA inhibition by IMER. Future perspectives and potential implications of this research are provided in a final concluding chapter.

CHAPTER 2. CONTINUSOUS FLOW IMMOBILIZED ENZYME REACTOR-TANDEM MASS SPECTROMETRY FOR SCREENING ACHE INHIBITORS IN COMPLEX MIXTURES

The following chapter was published in the journal *Analytical Chemistry* under the following citation:

Forsberg, E.M., Green, J.R.A., Brennan, J.D., **Continuous Flow Immobilized Enzyme Reactor-Tandem Mass Spectrometry for Screening AChE Inhibitors in Complex Mixtures**, Analytical Chemistry, **2011**, 83(13), 5230-5236.

The author conducted the majority of this research independently. An initial batch of acetylcholinesterase columns were prepared by James R.A. Green, which provided proof of concept data for sol-gel derived AChE columns using absorbance spectroscopy. The author synthesized the diglyceryl silane silica precursor and remaining sol-gel derived columns used in the study. The HTS facility provided the bioactive compounds for screening while the author prepared the mixtures for screening, developed the mass spectrometric methods and performed the data analysis on the IMER screen and IC_{50} curves. The final manuscript was drafted and completed by Dr. Brennan and the author.

Abstract

A method is described for identifying bioactive compounds in complex mixtures based on the capillary-scale monolithic enzyme-reactor columns for rapid screening of enzyme activity. A two-channel nanoLC system was used to continuously infuse substrate coupled with automated injections of substrate/small molecule mixtures, optionally containing the chromogenic Ellman reagent, through a sol-gel derived acetylcholinesterase (AChE) doped monolithic columns. This is the first report of AChE encapsulated in monolithic silica for use as an immobilized enzyme reactor (IMER), and the first use of such IMERs for mixture screening. AChE IMER columns were optimized to allow rapid functional screening of compound mixtures based on changes in the product absorbance or the ratio of mass spectrometric peaks for product and substrate ions in the eluent. The assay had robust performance and produced a Z' factor of 0.77 in the presence of 2% (v/v) DMSO. A series of 52 mixtures consisting of 1040 compounds from the Canadian Compound Collection of bioactives was screened and two known inhibitors, physostigmine and 9-aminoacridine, were identified from active mixtures by manual deconvolution. The activity of the compounds was confirmed using the enzyme reactor format, which allowed determination of both IC_{50} and K_I values. Screening results were found to correlate well with a recently published fluorescence-based microarray screening assay for AChE inhibitors.

Introduction

The discovery of new compounds that can modulate the function of key disease-related proteins is a fundamental requirement for the development of new therapeutics. The most common approach to identify such compounds is to perform high-throughput screening of up to several million discrete compounds using an automated assay. Common methods of high-throughput screening involve colourimetric or fluorometric assays run in a multi-well plate format.[155,156] Drawbacks to these methods include the need for chromogenic or fluorogenic reagents, the potential for interferences form compounds that absorb, fluoresce or quench fluorescence, the need for complex robotic liquid handling,[157] and an inability to apply these methods to complex mixtures, such as natural product extracts.[158] When spectroscopic analysis of compounds libraries are not feasible, either mass spectrometry (MS)[159-171] or HPLC-based assays[170,172] can be performed, but these are typically time consuming and generally not scalable to high-throughput.

Immobilized enzymes can be used for mixture screening in two main formats: 1) as immobilized enzyme reactors (IMERs);[15,173-176] 2) as affinity phases to isolate high affinity compounds from mixtures.[130,134,177-180] The enzyme is usually immobilized in a column, either through covalent bonding or avidin-biotin interactions. More recently, our group has demonstrated that enzymes can be entrapped into the pores of monolithic silica columns that contain both mesopores (~3 – 50 nm diameter) to retain protein and macropores (0.5 – 1 μm) to allow flow of liquid with low backpressure.[128,130,176,180] IMER columns can be interfaced to absorbance,[181] fluorescence[143] or MS-based

detectors[182] directly or interfaced to a second dimensional reversed phase liquid chromatography (RPLC) column to separate substrates and products prior to detection by one of these methods.[15,173] IMERs can also be interfaced in an off-line fashion to detection methods such as MALDI/MS,[183,184] or free enzymes can be used to catalyze reactions using immobilized substrates with MALDI/MS detection.[185-187]

Advantages of IMER-based screening include the ability to screen mixtures, the potential to reuse enzymes for multiple assays,[175,188] and the ability to incorporate an autosampler to allow for semi-automated screening. It is also possible to program different flow profiles into the pumps to allow for continuous variation of substrate and inhibitor levels to quantify inhibitor potency.[176]

While IMERs coupled to MS have been widely utilized for assessing the inhibition of enzymes by small molecules, few studies have evaluated the potential of IMER/MS/MS for screening of compound libraries. Issues such as assay reproducibility, tolerance to dimethylsulfoxide (DMSO), which is a common solvent for storing library compounds, and the ability to screen complex mixtures in an automated fashion remain to be addressed, particularly for capillary scale IMERs that utilize direct pumping of substrates and inhibitors via low flow nanoLC. In this study, an acetylcholinesterase (AChE) IMER assay is developed, characterized and used for automated screening of compound mixtures to identify inhibitors of AChE by monitoring the product concentration via absorbance or MS detection. The enzyme is immobilized within monolithic silica prepared via a sol-gel method.[130] AChE was chosen as the model enzyme owing to its relevance as a target for Alzheimer's disease therapeutics.[181,189-192] Use of this enzyme

also allows comparison to previous studies of AChE IMERs using covalently immobilized enzyme, reported by Bartolini *et al*.[181,193-195] Identification of bioactive mixtures using automated IMER assays is demonstrated using both absorbance-based detection (Ellman reaction)[196] and MS-based detection (multiple reaction monitoring (MRM) mode). Assay development focused on validating the robustness of mixture screening with compound libraries using the two modes of detection, assessing the effect of DMSO, and demonstrating automated screening of 52 randomly selected bioactive compound mixtures made from the Canadian Compound Collection. A comparison with a recently developed fluorescence-based microarray mixture screening assay[73] was also carried out to confirm the validity and sensitivity of the IMER assay. Identification and quantitative assessment of the potency of active compounds from inhibitory mixtures via manual mixture deconvolution and IMER screening is also demonstrated, showing the potential of the function-based solid-phase assay method for identification of active compounds in mixtures.

Experimental Section

Materials: Ammonium acetate, HEPES, acetylcholinesterase (AChE, from *Electrophorus electricus*, EC 3.1.1.7), acetylthiocholine iodide (ATCh), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), fluorescein, galanthamine, huperzine A and 10 kDa poly(ethylene glycol) (PEG) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Diglycerylsilane (DGS) was prepared using a modified synthesis protocol[197] described in detail in Supporting Information (SI). Other compounds used for mixture screening were

obtained from the Canadian Compound Collection. Fused silica tubing was purchased from Polymicro Technologies (Phoenix, AZ, U.S.). Distilled deionized water was from a Milli-Q Synthesis A10 water purification system. AChE was exhaustively dialyzed using Spectra/Por 6, 50 kDa dialysis tubing into 225 mM HEPES, pH 7.4. All other reagents were used as received.

Fabrication of AChE Columns: Columns were fabricated in a fashion similar to that described in our previous reports[128,130,176,180] to a final composition of 42.5 Units.mL^{-1} AChE, 0.5 g.mL^{-1} DGS, 5% (w/v) 10 kDa PEG, 56 mM HEPES at pH 7.4. Columns were cured for 5 days prior to cutting 5 cm segments and equilibrating as required (see SI).

Assay Validation and Reproducibility: Assay validation was done with a series of positive and negative controls using absorbance detection and was compared to MS detection, using the LC-MS configurations shown in Figure 2.1. Detailed LC-MS settings, including flow rates and MS parameters, are provided in the SI. Negative controls contained 100 μM ATCh, 2% (v/v) DMSO and, when used with absorbance detection, 100 μM DTNB. Positive controls for the absorbance assay used no ATCh, but contained 2% (v/v) DMSO and 100 μM DTNB. For MS assays, positive controls used an AChE-doped column, to which a series of "no substrate" samples were injected, and a blank column where ATCh samples were injected. Data from the two runs was compiled to determine the minimum product to substrate signal ratio (P/S) achievable in the assay. Negative controls were the same as were used in the absorbance assay, though for MS assays there was no DTNB present. The Z′ factor, which is a measure of assay

reproducibility, was determined using the average control signal levels, μ, and standard deviations, σ, for positive (+) and negative (-) controls, as per equation (1).[198]

$$Z' = 1 - \frac{(3\sigma_+ + 3\sigma_-)}{|\mu_+ - \mu_-|} \quad (1)$$

Mixture Screening: Mixtures were screened using the autosampler system configuration shown in Figure 2.1A with the MS detector operated in multiple reaction monitoring (MRM) mode. In this configuration, the mobile phase in Channel A and the test mixtures contained an identical concentration of substrate (ATCh), DTNB, and DMSO, with mixtures introduced through an autosampler loop. Channel B contained MeOH, which was added post-column as a MS make-up flow to aid in ionization. A total of 1040 bioactive small molecules were initially obtained as 1 mM stocks in DMSO. Each mixture was formed by diluting 20 randomly selected compounds to a final concentration of 1 μM each, resulting in a final solution containing 100 μM ATCh, 20 mM NH_4OAc, pH 7.0 and 2% (v/v) DMSO. The mobile phase had an identical solution composition to avoid any variations in mobile phase composition during injection of mixtures onto the column. Positive controls contained only 1 μM galanthamine in the above buffer solution, while negative controls did not contain any inhibitory compounds. Columns were first equilibrated with mobile phase to obtain an initial P/S signal ratio based on turnover of substrate in the mobile phase to product ions by immobilized AChE. The activity of each mixture was measured by determining the P/S signal ratio in the presence of the mixture

and then normalizing this to the initial P/S signal ratio prior to injection of the mixture, as shown in equation (2):

$$\%activity = \frac{\left(P/S\right)_{sample}}{\left(P/S\right)_{initial}} \times 100 \quad (2)$$

Determination of IC_{50} and K_I Values: An alternative pump configuration, shown in Figure 2.1B, was used for quantitative determination of inhibition constants. In this case, a solution of 100 μM ATCh was loaded into Channel A, while Channel B was loaded with 100 μM ATCh plus either 3 μM of galanthamine or 1 μM of huperzine A (all present in 20 mM NH_4OAc, pH 7.0 with 2% (v/v) DMSO). IC_{50} values were obtained by altering the ratio of flow in the substrate and substrate+inhibitor channels in a stepwise fashion while maintaining a combined flow rate of 10 $\mu L.min^{-1}$. In the LC method, each mobile phase ratio remained constant for 10 minutes to allow for an equilibrium condition to be achieved within the column. Data collected from a 4 minute window were averaged to give each data point. The raw data was normalized by letting the maximum product concentration in the absence of inhibitor correspond to a relative activity of 100% and the signal of only channel A without the AChE column present correspond to zero activity. IC_{50} values were obtained from the point where the relative activity decreased to 50% of its initial value. The inhibition constant (K_I) was determined from the IC_{50} value as described by Cheng and Prusoff:[199]

$$K_I = \frac{IC_{50}}{1 + \frac{[S]}{K_M}} \tag{3}$$

where [S] is the concentration of substrate and K_M is the Michaelis constant. All data was fit using Sigma Plot graphing software.

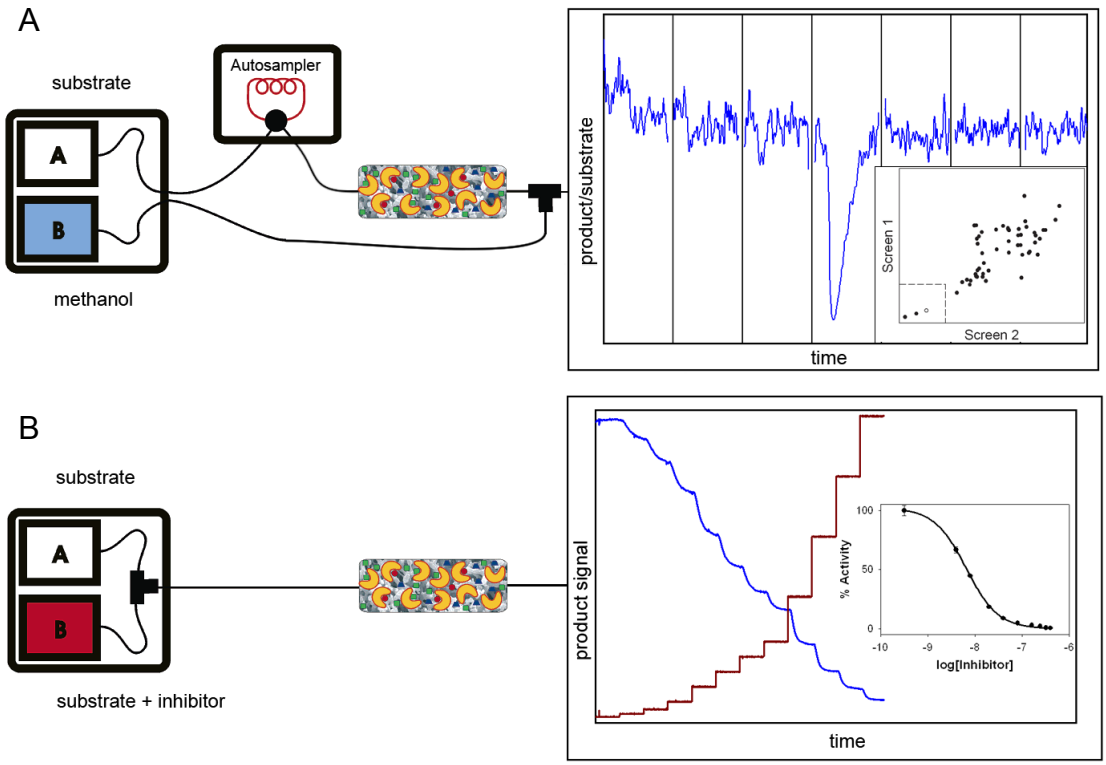

Figure 2.1. (A) Method for primary mixture screening using a capillary scale immobilized enzyme reactor column interfaced to a MS/MS detector. Mobile phase in Channel A and samples contain an identical concentration of substrate (ATCh), DTNB, and DMSO, while inhibitors are introduced through an autosampler loop. Channel B contains MeOH which is added post-column as a MS make-up flow. In this example, mixture **4** contains an inhibitory compound. (B) Method for determining IC_{50} values using inhibitor infused directly from pump B. In both cases, increases in the concentration of an inhibitor cause a reduction in the signal of the product.

Results and Discussion

Column Optimization: Columns were optimized for maximal protein retention, enzymatic activity and nanoLC-MS/MS compatibility. The fabrication method was based on previous sol-gel entrapment methods, with pH varied to obtain optimal columns.[180] Columns fabricated at pH 7.4 provided optimal enzyme retention and activity; the properties of such columns including pore morphology, relative enzyme leaching and enzyme activity, and kinetic parameters for entrapped AChE, are provided in the SI.

IMER assays were initially performed using both absorbance and MS detection modes. Absorbance methods allow a wider range of buffers, but suffer from potential interferences from strongly absorbing compounds, while MS/MS methods can be used in cases where colorimetric reagents are not available, but must utilize low ionic strength buffers that are compatible with the electrospray process. Prior to performing a mixture screen, the automated delivery of reagents to the column via the autosampler and the MS parameters were optimized to allow for a rapid and reliable assay. Our studies showed that AChE retained high activity when using 20 mM ammonium acetate as a buffer, though activity was markedly reduced, to a level of ~20% of the initial value, upon introduction of 2% (v/v) DMSO. However, the enzyme activity remained stable at this level for several hours in the presence of DMSO, demonstrating that DMSO inhibited rather than degraded the enzyme, in agreement with previous reports on the effect of DMSO on AChE.[200] MS signal intensity was also somewhat reduced upon introduction of DMSO (by 60%), but again was stable so long as the concentration of DMSO remained constant.

Once suitable MS-compatible buffer conditions were obtained, suitable parent and daughter ions were identified for ATCh, thiocholine (TCh), galanthamine and huperzine A (test inhibitors). MRM transitions for each species and the optimized MS conditions are provided in the SI. In all cases it was possible to obtain very high signals with essentially no background interference and no overlap of signals between species. These species could be monitored simultaneously using MRM mode, allowing for analysis of product/substrate ratios as a function of inhibitor concentration. For qualitative screening purposes it is sufficient to monitor the P/S signal ratio directly and assign "hits" based on a threshold P/S signal level, as described below.

Z′-Factor. Figure 2.2 shows Z′ plots obtained using both absorbance and ESI-MS/MS detection. Both analyses gave a Z′ factor of 0.77, indicating a highly reproducible assay and demonstrating successful transfer of the assay from the absorbance-based to the MS-based platform. A second point from Figure 2.2 is that the P/S ratio is highly reproducible as a function of inhibitor concentration. The reproducibility and large assay window clearly show that the assay is very robust. Another point to note is that inhibitors with K_I values in the high nanomolar and greater range show full signal recovery after equilibration, showing that the optimized assay conditions provide sufficient time between injections to allow removal of moderately potent competitive inhibitors from the enzyme when substrate was used in the wash cycle. We note that the presence of potent compounds with very slow off-rates may lead to significantly slower recovery times, and this could potentially lead to carry-over effects from one mixture to another. Running a data dependent MS method that only injects the next sample after a minimum threshold

P/S level is regained could be used to minimize carry-over effects from compounds with slow off-rates, though such a method was not required in this work.

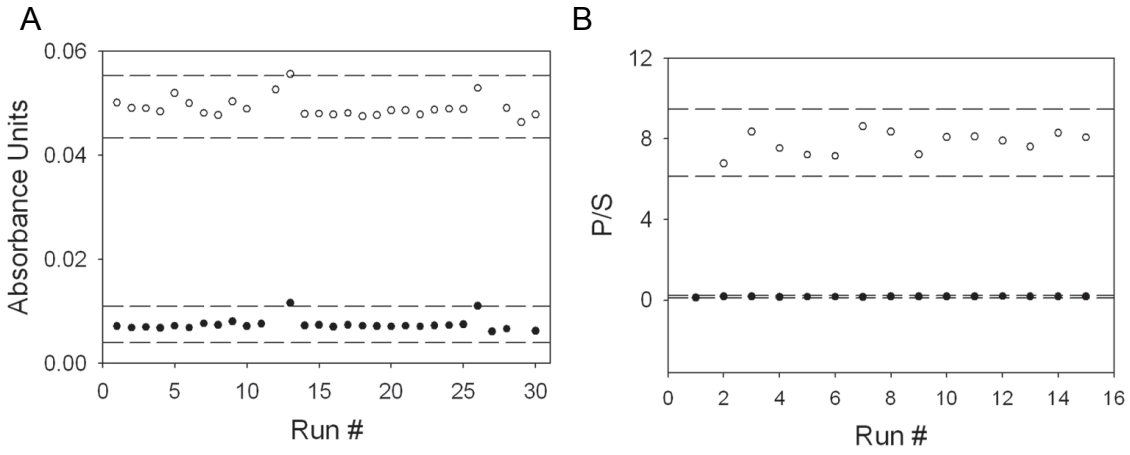

Figure 2.2. Assay validation for automated inhibitor screening using: (A) Z' plot for absorbance-based detection of 5 cm AChE columns with alternating injections of positive controls containing no substrate, and negative controls containing 100 μM substrate; (B) Z' plot for ESI-MS/MS detection using a blank column with injections of substrate to determine maximal substrate signal and AChE column with no substrate to obtain minimum product signal. Z' was calculated to be 0.77 for both assay detection modes.

Automated Mixture Screening. Figure 2.3A shows the results of two separate automated screens of mixtures using the IMER coupled to ESI-MS/MS. The mixtures contained only 1 μM of each test compound to minimize the potential for ion suppression and to further assess the sensitivity of the assay and provide more stringent screening conditions. The assay points for mixtures generally fall on a diagonal, and demonstrate good reproducibility between duplicate assays. Although DMSO was an interferant in the assay, it was still possible to easily identify mixtures with active compounds present, as well as the galanthamine control compound, which always showed at least a 4-fold decrease in the P/S ratio relative to the blanks that contained DMSO.

The duplicate assay utilized a 15 μL injection loop (6 column bed volumes) to ensure a sufficient quantity of inhibitor was exposed to the enzyme active sites to reach equilibrium. Under these conditions test mixtures were injected over a period of 3 min followed by a 15 min washing step prior to injection of the next test mixture, resulting in an assay time of 18 min per mixture. Given that each mixture contained 20 compounds, assay throughput was approximately 1 compound per minute, which is 10-fold higher than the throughput of our previously reported IMER/MS assays.[176] While this assay rate does not meet the criteria for a "high-throughput" assay (>10,000 samples per day), it does provide medium throughput and serves to demonstrate the principle of the automated mixture screening protocol. Increased throughput could be easily achieved by further increasing the mixture complexity and flowrate, though issues with ion suppression and short equilibration times, respectively, would limit the extent to which these parameters could be adjusted. Furthermore, the use of 20 compounds per mixture provided a useful compromise between sample throughput and ease of mixture deconvolution.

Inhibitor Identification. Those mixtures that showed a decrease in the P/S signal ratio to below 50% were separated into their individual components and rescreened. Figures 2.3B and 2.3C show two duplicate plots obtained from individual compound screening of mixtures 19 and 46, respectively. From these mixtures, two potent inhibitors were identified, each reducing the AChE activity to well within the acceptable hit window. The compounds responsible for inhibition were identified as physostigmine (mixture 19, K_I = 15 nM)[201] and 9-aminoacridine (mixture 46, K_I = 90 nM).[171] The single component

screens showed a small spread very close to 100% activity for all components other than the positive control and the identified inhibitors. The reproducibility of single compound screening is much greater than for mixture screening, as seen by the variation in mixture inhibition in the 1040 compound screen in Figure 2.3A. This is mainly due to the reduced DMSO content (0.1%) for screening of single compounds, which resulted in higher initial enzyme activity, and possibly also to lower ion suppression resulting from injection of only one compound per injection.

An important point to note from the above assays was the ability to identify a total of two bioactive compounds using a total of only 120 injections (20 mixtures + 40 individual samples in duplicate). Duplicate screening of each individual compound would have required 2080 injections, thus the screening and deconvolution of mixtures required less than 6% of the time that would be needed to screen individual compounds. It is also noted that the "hit rate" for the screen was slightly less that 0.2% (2 compounds of 1040), while that of the mixture screen was ~4% (2 mixtures of 52), resulting in a better hit rate and more rapid identification of bioactive compounds when using mixtures.

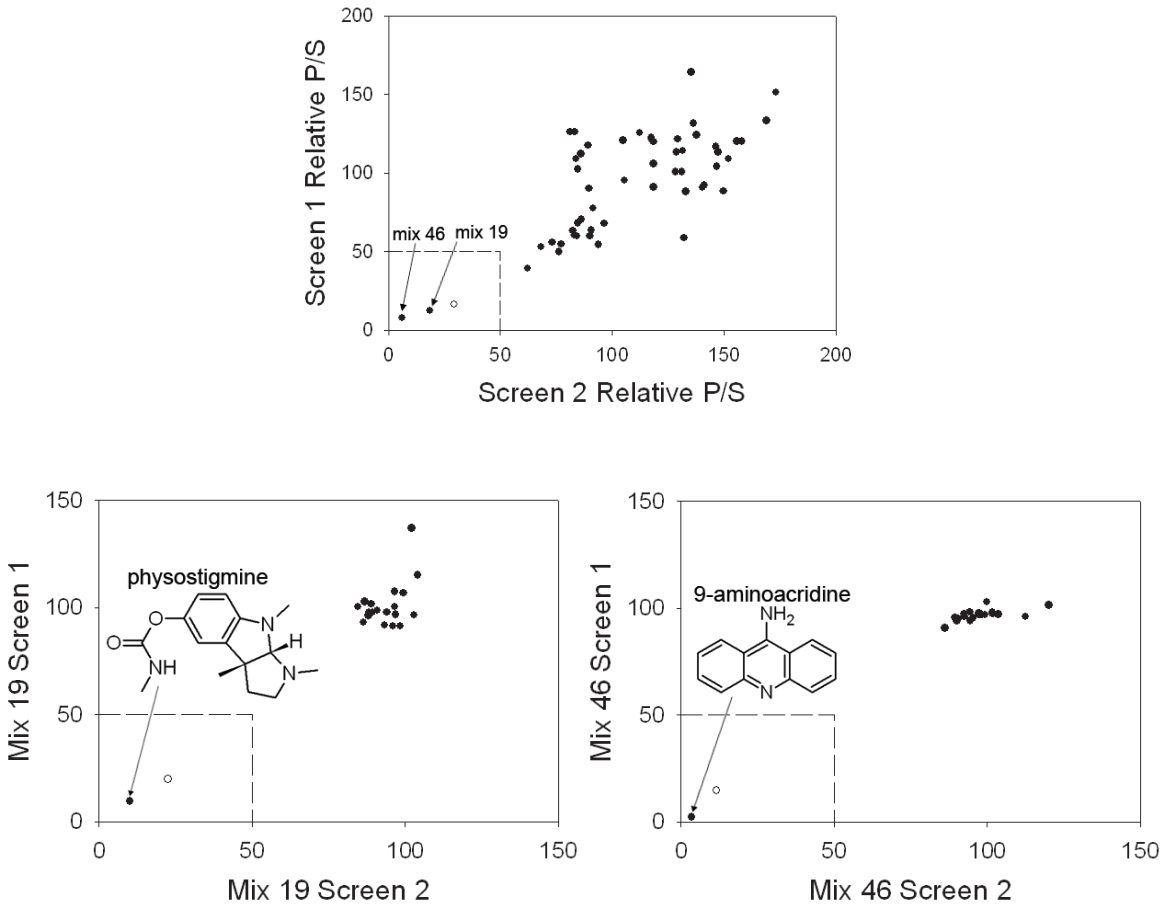

Figure 2.3. (A) duplicate plot for 1040 bioactive compound screen consisting of 52 mixtures with 20 compounds in each, and the control galanthamine. (B) and (C) show duplicate plots for individual components of hit mixtures.

Quantitative Binding Analysis by IMER. Once a ligand is identified as a "hit", it is important to perform secondary screening to ensure that the ligand does indeed inhibit the enzyme and to determine the inhibition constant. Figure 2.4 shows the IC_{50} curves for galanthamine and huperzine A obtained using an on-line continuous flow enzyme reactor assay with absorbance detection. Figure 2.1B shows the assay format, where the amount of inhibitor is varied simply by altering the ratio of flowrate of pumps A and B, with both

pumps having identical amounts of the substrate ATCh and the chromogenic reagent DTNB, but with inhibitor present only in pump B. The response shows that the absorbance as a function of time decreases due to increased inhibition as the concentration of inhibitor increases. The recovered IC_{50} and K_I values of 480 nM and 270 nM for galanthamine were in good agreement with those obtained in solution (460 nM and 230 nM) and the K_I was also in reasonable agreement with the published K_I value of 280 nM.[202] Analysis of the huperzine A data gave an IC_{50} of 46 nM and K_I of 26 nM, while data obtained in solution gave values of 6.5 nM and 3.3 nM, respectively. The higher inhibition constant obtained for the solid-phase assay of huperzine A likely reflects electrostatic interactions and mass transfer limitations within the stationary silica phase, which reduce the concentration of inhibitor that reaches the entrapped enzyme and thus requires a higher solution concentration for inhibition.[203] Despite this, the results obtained for entrapped AChE are still in reasonable agreement with solution data, showing that the chromatographic method can be used not only for primary screening, but also for quantitative determination of inhibition constants. Hence, the use of the IMER method provides kinetic and inhibition data that are of sufficient accuracy to allow the method to be used for both primary screening and quantitative analysis of enzyme inhibition.

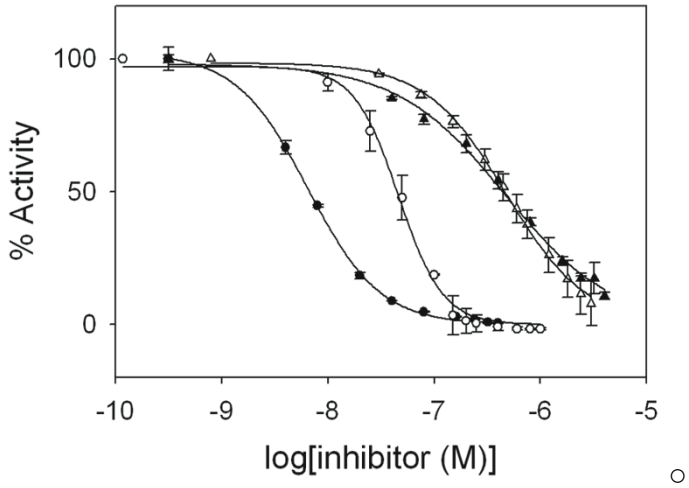

Figure 2.4. Determination of IC_{50} values on-line using continuous flow enzyme reactor mode. The IC_{50} curves are recovered upon taking an average absorbance value after equilibrating the column with a new concentration of inhibitor. The recovered IC_{50} values are in good agreement with those obtained in solution. Galanthamine on column (▲) IC_{50} = 480 nM, K_I = 270 nM; galanthamine in solution (Δ) IC_{50} = 460 nM, K_I = 230 nM; huperzine A on column (●) IC_{50} = 46 nM, K_I = 26 nM; huperzine A in solution (○) IC_{50} = 6.5 nM, K_I = 3.3 nM.

It is interesting to compare our findings to those of Bartolini *et al.*, who have also done a significant amount of work on AChE IMERs utilizing absorbance detection using the Ellman reagent.[173,181,193,195] In their work, the enzyme was covalently immobilized onto CIM® disk monolithic columns via a Schiff base method, resulting in ~0.22 U of activity per column (bed volume of 0.34 mL) vs. 0.11 U of activity per column (2.45 μL bed volume) for our capillary scale columns. Their assays utilized a more rapid flowrate of 1.0 mL.min^{-1} relative to our flowrate of 20 μL.min^{-1}, but in their case substrate and inhibitor samples were injected as discrete 10 μL aliquots into a buffer flow stream (much lower than the column bed volume), while our 15 μL injections were sufficient to reach equilibrium on the column (6 bed volumes). The method utilized by Bartolini *et al.*

allowed for determination of IC_{50} values from known inhibitors and required only 5 min per sample, but up to 100 min for recovery of full enzyme activity, where in our assay recovery was much faster owing to the use of substrate to accelerate dissociation of inhibitors. The authors did not apply their IMERs to screening of mixtures, as was done in our work, but they did note that this was possible.

Comparison to Microarray Screen. Recently, our lab published an AChE microarray screening method using a fluorogenic BODIPY dye to detect the presence of inhibitors.[73] The assay was used to screen the same set of bioactive compounds as was used for the IMER assay. As a way validate both assays, a comparison between the fluorogenic microarray assay and the IMER-ESI-MS/MS assay was performed. A duplicate plot is presented in Figure 2.5 that compares the average activity from each respective assay for each mixture and shows that mixture 19 is identified as a hit in both assays, while mixture 46 is identified as a hit only in the IMER assay. The inability of the microarray assay to identify mixture 46 as a hit is most likely the result of the fact that this mixture, which contains 9-aminoacridine, is fluorescent (λ_{ex} = 400 nm, λ_{em} = 430 nm), and thus is likely to cause a false negative result. Thus, while the fluorogenic BODIPY microarray assay is extremely versatile and can be applied to a variety of compound libraries in various buffer systems, the IMER-ESI-MS/MS assay appears to be more resistant to interferences resulting from fluorescent compounds. The IMER assay also benefits from requiring no incubation time of mixtures and substrate with the immobilized enzyme and reuses the same 2.45 μL bed volume of AChE for all the mixtures.

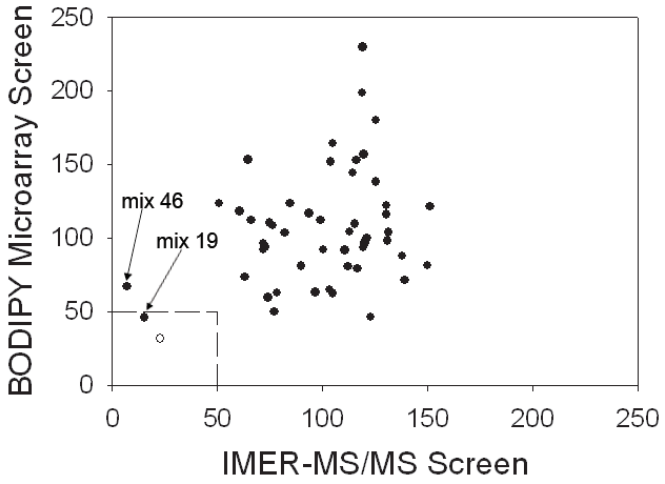

Figure 2.5. Comparison of fluorogenic BODIPY AChE microarray screen of 52 mixtures with IMER-ESI-MS/MS screen of the same mixtures. Both assays were successful at identifying the mixture containing physostigmine as a hit below 50% activity, while one mixture was identified as a hit in the IMER assay but not in the microarray assay.

Conclusions

Interfacing of enzyme reactor columns to tandem MS provides a rapid method for function-based assessment of enzyme inhibition and is amenable to direct screening of enzyme inhibitors within mixtures. The use of MS for assessing enzyme activity is highly versatile, and avoids the need for labels. The use of an immobilized enzyme column allows re-use of the enzyme for multiple assays, which not only saves on reagent costs but also provides an unprecedented internal control in that the level of enzyme is consistent for all assays.

The screening assay has excellent reproducibility (Z'=0.77) even when moving from an absorbance-based assay to an ESI-MS/MS assay that requires a volatile buffer. The ability to detect protein-binding ligands in mixtures even in the presence of 2% (v/v)

DMSO was demonstrated, which makes the assay amenable to standard compound libraries. The use of short columns and higher flowrates, relative to our previous IMER studies, provided increased throughput and decreased assay time. The IMER assay was capable of identifying two mixtures containing AChE inhibitors and could successfully deconvolute the mixtures to identify the inhibitor. Quantitative analysis of inhibitors in an automated manner provided inhibition constants that were in good agreement with solution data. Further studies will examine methods to further increase mixture complexity and reduce assay time per mixture and will evaluate alternative methods to deconvolute mixtures in an effort to make the method amenable to screening of complex natural product extracts.

CHAPTER 2. SUPPLEMENTARY INFORMATION

Experimental Details

Fabrication of AChE Columns

Diglycerylsilane Synthesis. Tetramethylorthosilicate, (*Sigma Aldrich*, 24.2 mL, 0.164 mol) is mixed with glycerol (*Fisher Scientific*, 25.4 mL, 0.348 mol), heated and mixed at 105ºC under N_2 until homogenous, then 120ºC for 18 hours. Residual methanol is distilled off at 130ºC for another 2 hours, followed by drying under vacuum at 140ºC. It is imperative that anhydrous conditions are maintained throughout the synthesis and confirmation of product by ^{29}Si- and ^{13}C-NMR is performed.

Column Fabrication: 5 mL of dialyzed AChE (1700 Units.mL^{-1}, where 1 U = 1 mmol.min^{-1} of product formed) mixed with 45 mL with 225 mM HEPES buffer either pH 7.0 or 7.4 and 50 mL aqueous 20% (w/v) of 10 kDa PEG. Sols were prepared by sonicating DGS with water (1 g + 1 mL) at 0 °C for 20 – 25 min followed by filtration through a 0.2 μm filter. 100 mL of the resulting sol was rapidly mixed with AChE/PEG solution to give a final concentrations of 42.5U/mL AChE, 0.5 g/mL DGS and 5% (w/v) PEG in 56 mM HEPES. The sol solution was injected into 80 cm of 250 μm i.d., 360 μm o.d., polyimide coated fused silica tubing that was previously cleaned using 3 – 4 bed volumes of 1 M NaOH, 1M HCl, Milli-Q H_2O and EtOH, then coated with 2% (v/v) APTES in EtOH. The resulting sol underwent phase separation in 55 s and gelled in 65 s. After gelation, capillary ends are submerged in 100 mM HEPES and secured for storage. Columns were aged for a minimum of 5 days to achieve a stable internal structure.

Column Handling. Prior to experiments, a fresh 5 cm column segment (2.45 μL internal volume) was equilibrated off-line with mobile phase from Channel A (100 mM HEPES or 20 mM ammonium acetate, pH 7.0), to remove free PEG and glycerol. New columns were connected to the pump using 50 μm i.d. fused silica tubing using Upchurch Microtight unions (S.P.E., North York, ON). Several bed volumes of mobile phase were passed through the column at 0.5 $mL.min^{-1}$ before increasing the flow rate to 2, 5 and 10 $mL.min^{-1}$ for experiments. For IMER/MS studies columns were attached directly to the electrospray ionization source of the Q-Trap mass spectrometer with 50 μm i.d. fused silica tubing. When exchanging mobile phases within the pumps, the column was removed from the system and connected top to bottom with a buffer filled capillary. The column fittings were not adjusted or removed from the column after the initial washing step.

Mixture Screening

Michaelis-Menten Kinetics. The absorbance system was first calibrated using varying concentrations of thiocholine (Figure S2.1A), and showed a linear relationship over a concentration range of 0 > 350 mM and non-linear up to 500 mM. As a result, a non-linear, 2^{nd} degree polynomial curve was used to fit the data. The concentration of product formed was determined at various substrate concentrations and flowrates, and the concentration of product was divided by the contact time of the substrate on the column to determine the amount of product formed per minute per column. This value was plotted against substrate concentration at each flow rate to give Michaelis-Menten curves

(Figure S2.1B). GraphPad Prism v4 software was used to determine the catalytic properties of the entrapped enzymes.

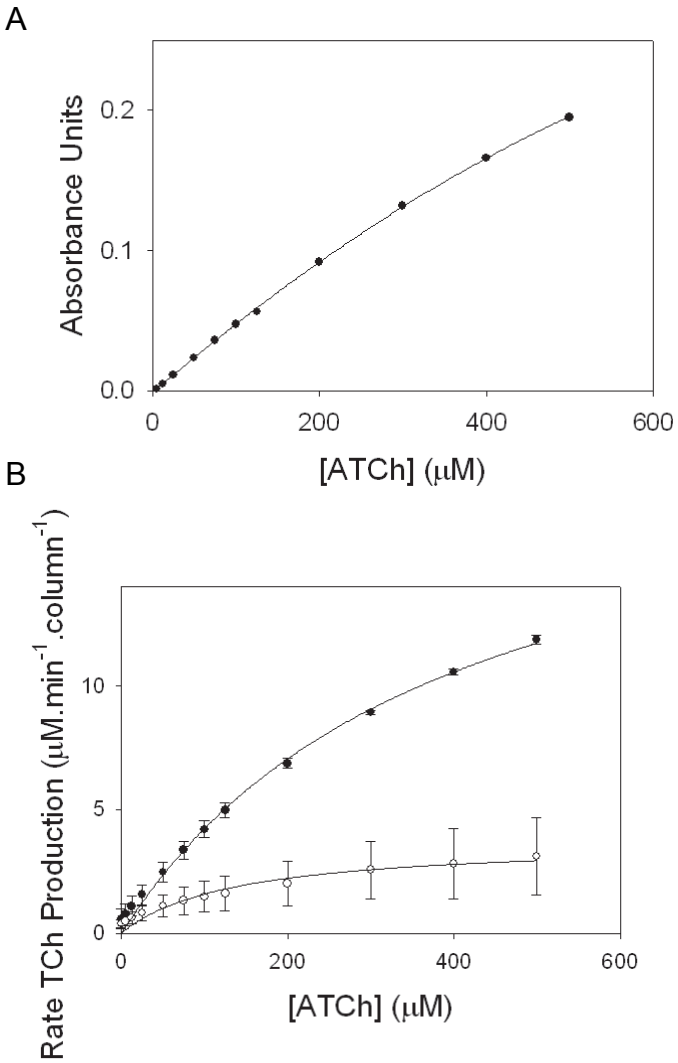

Figure S2.1. Figure showing (A) absorbance calibration of signal vs. [P], (B) signal vs. [S] (Michaelis Menten plot) at 5 and 10 μL/min to show optimization of flow rate conditions.

LC/MS Settings. An Eksigent 1D-nanoLC was used for mobile phase delivery to either a GL Sciences 701 UV-Vis detector equipped with a 6 nL flow-cell (40 μm i.d., 4 mm path

length), or an AB/Sciex Q-Trap Mass Spectrometer with a Turbospray ion source. An Eksigent AS-1 autosampler was fitted with a 250 μL withdrawal syringe and a 15 μL sample loop of 250 μm i.d. fused silica tubing. Larger volumes can be loaded directly into the 5mL Eksigent pump reservoir for determination of IC_{50} values. Mobile phase delivery was controlled by Eksigent nanoLC software v 2.08. Mobile phases were run directly into the absorbance detector, or were mixed with methanol as make-up flow prior to introduction to the MS Mass spectrometer control and data acquisition of analyte signals was done using Analyst 1.4.1 software. Precursor-product ion pairs were followed using MRM in positive ion mode under the following conditions: Curtain Gas = 45.0, Collision gas = medium, Ion Spray Voltage = 5500 V, Temperature = 175 °C, Ion Source Gas 1 = 40.0, Ion Source Gas 2 = 40.0. Specific MS/MS parameters for each ion pair are provided in Table S2.1. The total scan time was 1 second per point.

Table S21. MS Settings for acetylthiocholine, thiocholine, galanthamine and huperzine A.

Species	Precursor ion (m/z)	Fragment ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
ATCh	162.3	103.1	30	3	17	2.4
TCh	120.0	60.0	35	4	24	2.0
Galanthamine	288.4	213.2	45	5.5	28	3.5
Huperzine A	243.2	226.3	75	5	29	3

Mixture Sample Preparation. Each mixture contained 20 compounds, each diluted to 1 mM in 20 mM ammonium acetate buffer containing 100 mM ATCh and 2% (v/v) DMSO. Positive controls contained 100 mM ATCh, 2% (v/v) DMSO spiked with 1 mM

galanthamine and negative controls contained only 100 mM ATCh, 2% (v/v) DMSO. The DMSO was included to mimic the solvent composition that would be present in the compound library. After suitable column equilibration (see above), sample mixtures were infused from the 15 mL autosampler loop at a total flowrate of 5 $mL \cdot min^{-1}$ and mixed with 5 $mL \cdot min^{-1}$ MeOH from channel B, giving a total flow of 10 $mL \cdot min^{-1}$ through ESI-MS. The LC method was set to pre-flush the column for one minute prior to injection and flush the column for 18 minutes after injection.

Results and Discussion

Fabrication of AChE Columns

Column Optimization. Key parameters affecting column morphology are the concentration of PEG and the fabrication pH, with macropore size and volume increasing with PEG concentration and decreasing as pH increases over the range of 6.9 – 7.6. AChE columns were prepared with a PEG concentration of 5% (w/v) at pH 7.0 and 7.4 to evaluate the effect of morphology on column performance and enzyme activity. Figure S2.1 shows the response of AChE bioreactor columns made with 5 % (w/v) PEG at pH 7.0 and 7.4 upon infusion of ATCh/DTNB with and without the inhibitor galanthamine. The data clearly show that the columns formed at pH 7.4 have higher initial activity, and a more stable signal over time.

LC/MS Optimization. Moving from absorbance-based detection to mass spectrometric detection required optimization of a volatile buffer mobile phase. Inclusion of DMSO in the mobile phase was also necessary for application to the bioactive compound library.

Enzymatic activity was observed over many repeated injections of 2.5 μM galanthamine to confirm stability (Figure S2.2). AChE activity decreased by 20% when changing from 100 mM HEPES to 20 mM NH_4OAc, by 30% when moving to 20 mM NH_4OAc with 0.1% DMSO and by 60% when moving to 20 mM NH_4OAc with 1% DMSO. Signal stability was good over repeated inhibitor injections indicating the enzyme is not degrading significantly over long periods of time.

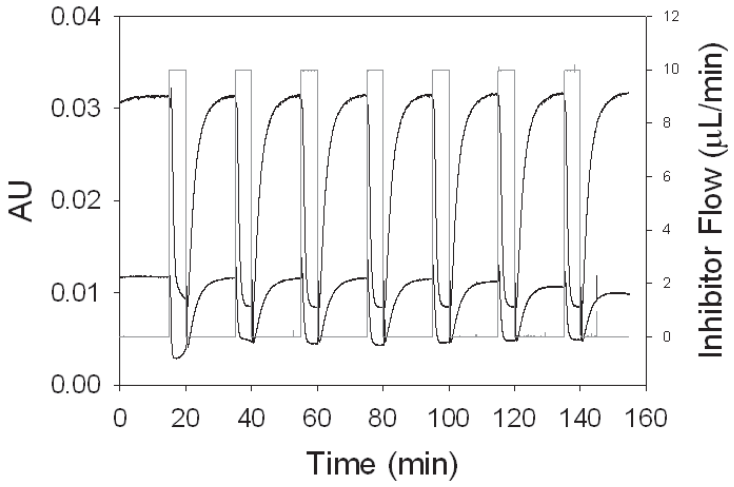

Figure S2.2. Effect of column fabrication pH on the on-line inhibition of immobilized AChE using absorbance detection. Channel A contains 100 μM ACh/DTNB; Channel B contains 2.5 μM galanthamine in 100 μM ACh/DTNB. Flowrate is 10 μL/min, column is 10 cm long, and enzyme loading is 0.2 Units. Buffer is 20 mM NH_4OAc buffer at pH 7.0. Top trace shows the response for columns formed at pH 7.4; bottom trace shows the response for columns formed at pH 7.0. The grey line shows the infusion profile with the flow rate from channel B shown on the right side of the plot.

Column Performance (K_M). The performance of columns prepared at pH 7.4 was evaluated in terms of catalytic activity of the entrapped enzyme as a function of flowrate and the long-term stability under different storage conditions. Table S2.2 shows the

Michaelis-Menten parameters for the AChE entrapped in columns as a function of the flowrate. In this case, the assay was performed using absorbance-based detection to avoid any issues with ion suppression when using MS-based detection, which can make calibration difficult. The Michaelis equation (S1) was used to calculate the maximum turnover rate (apparent V_{max}) and apparent K_M (Michaelis-Menten constant), where [S] is the substrate concentration and v is the observed reaction rate:

$$v = \frac{V_{max}[S]}{K_M + [S]} \quad \text{(S1)}$$

Two points are evident from Table S2.2. Firstly, the V_{max} decreases and the K_M value increases upon entrapment, regardless of flowrate. These trends are the result of limitation in the transport of substrate to the immobilized enzyme such that the enzyme is operating under diffusion controlled kinetics rather that enzyme-controlled kinetics. The second key point is that the reduced apparent V_{max} is likely the result of significant leaching of the enzyme, a fraction of inaccessible enzyme, and/or particularly large diffusion limitations owing the very low flow rates employed. There is no significant increase in backpressure upon increase of flow rate indicating pressure driven diffusion is less of a factor in this column composition. The decrease in apparent K_M with flow rate reflects an increase in the affinity of the substrate for the enzyme, however, it is more likely that the overall affinity remains relatively unchanged but the decrease in apparent V_{max} gives rise to the apparent increase in K_M.

Table S2.2. Kinetic parameters of AChE bioreactor column fabricated with 5% PEG and pH 7.4. Solution value is given as reference.

Flow Rate (μL/min)	Backpressure (psi)	K_M (μM)	V_{max} (μM.min^{-1}.column^{-1})
10	100	130±30	4±2
5	50	400±40	21±1
Solution	-	120	-

Mixture Screen Reproducibility. Duplicate data is sufficient for qualitatively determining potent hit mixtures, however it is useful to determine the reproducibility of the screening method under more scrupulous conditions. A selection of mixtures seen in Figure S2.3, including the two hit mixtures, were repeated in pentuplicate to illustrate the reproducibility of the assay with larger mixture complexity. The relative standard deviation (RSD) for all samples was ≤15%, except mixture 19 which had a RSD of 18%. Data from the hit mixture 19, containing the slow off rate inhibitor physostigmine, was obtained using 3 different bioaffinity columns all from different batches and was found to have higher variability due to column-to-column variation and the effect of slow P/S signal recovery on subsequent samples. Overall, the low RSD for the selected samples illustrates the excellent reproducibility of the IMER screening assay for reliably identifying protein-binding ligands in complex mixtures.

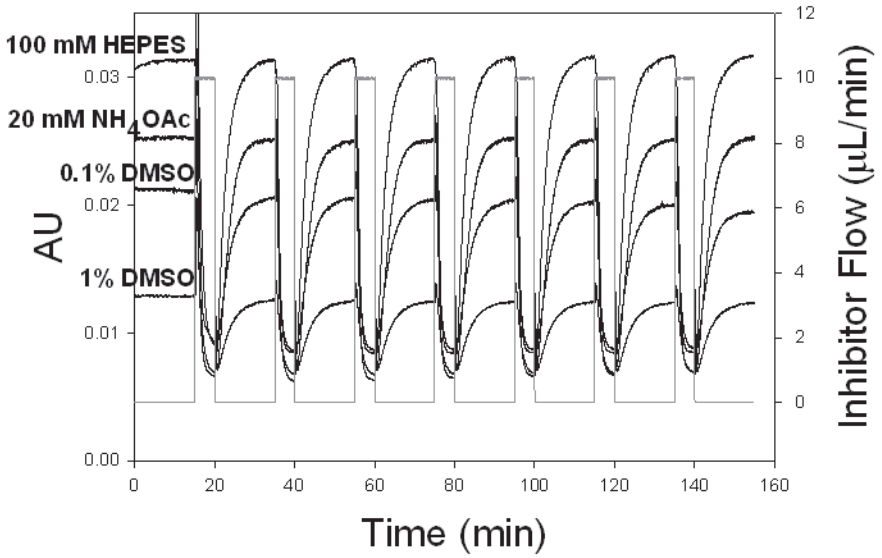

Figure S2.3. Optimization of LC/MS mobile phase for mixture screening using absorbance detection. Channel A contains 100 μM ACh/DTNB; Channel B contains 2.5 μM galanthamine in 100 μM ACh/DTNB. Top trace depicts mobile phase consisting of 100 mM HEPES, below showing 20 mM NH_4OAc, followed by addition of 0.1% DMSO and then 1% DMSO. Flowrate is 10 μL/min, column is 10 cm long, and enzyme loading is 0.2 Units. Buffer is 20 mM NH_4OAc buffer at pH 7.0. The grey line shows the infusion profile with the flow rate from channel B shown on the right side of the plot.

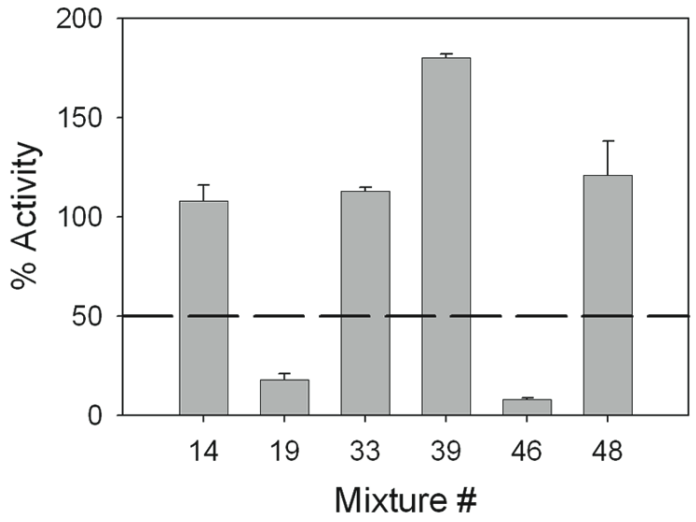

Figure S2.4. Pentuplicate screen of selected mixtures, including the two hit mixtures. The hits are clearly shown well below the dashed 50% cut-off line for determining hit mixtures. The RSD for each sample is extremely low, except for the hit mixture 19, which was tested on three different columns, each from a different batch.

CHAPTER 3. BIO-SOLID PHASE EXTRACTION / TANDEM MASS SPECTROMETRY FOR IDENTIFICATION OF BIOACTIVE COMPOUNDS IN MIXTURES

The following chapter was published in the journal *Analytical Chemistry* under the following citation:

Forsberg, E.M., Brennan, J.D., **Bio-Solid Phase Extraction / Tandem Mass Spectrometry for Identification of Bioactive Compounds in Mixtures**, Analytical Chemistry, **2014**, 86(16), 8457-8465.

The author conducted the majority of this research. The bioactive compound library was the same set of compounds used for the previous screen performed in Chapter 2 and was provided by the HTS facility. Richard Hodgson performed proof of concept experiments for development of ADA columns, however, the author performed materials optimization for enzyme entrapment for this study. The author prepared the mixtures for screening, developed the mass spectrometric methods and performed the data analysis on the IMER screen, IC_{50} curves, bioSPE protocol optimization and inhibitor elution analysis. The final manuscript was drafted and completed by Dr. Brennan and the author.

Abstract

We describe a two-step column-based bioassay method with tandem mass spectrometric detection for rapid identification of bioactive species in mixtures. The first step uses an immobilized enzyme reactor (IMER) column interfaced to an electrospray ionization mass spectrometer (ESI-MS) to identify mixtures containing bioactive compounds (i.e., enzyme inhibitors), while the second step uses bioselective solid-phase extraction (bioSPE) columns to isolate compounds from "hit" mixtures, which are then identified on-line by data-dependent ESI-MS. IMER columns were prepared by entrapment of adenosine deaminase (ADA) into sol-gel derived monolithic silica columns, and used to perform a primary IMER screen of mixtures prepared from a bioactive library, which resulted in four apparent hit compounds. Such columns did not provide sufficient binding site density to allow bioSPE, and thus a new column format was developed using ADA that was covalently immobilized to monolithic silica capillary columns, providing ~500-fold more protein binding sites than were present in columns containing entrapped proteins. Using the covalently linked ADA columns, bioactive mixtures identified by IMER were infused until a maximum total ion current was achieved, followed by washing with a buffer to remove unbound compounds. A harsh wash with 3% acetic acid eluted the strongly bound ligands and the resulting peak triggered data dependent MS/MS to identify the ligand, showing that two of the apparent hits were true ADA inhibitors and demonstrating the ability of this method to rapidly identify bioactive compounds in mixtures.

Introduction

The use of solid-phase assays is well established for small molecule screening, and includes microarray, microwell plate and chromatographic formats.[204] Among these, column based assays are a key format for screening of mixtures,[13,16,143,205-208] although microarray methods have been used as well.[73,209] Chromatographic approaches are generally either functional, as is the case for immobilized enzyme reactors (IMER)[128,143,206,210-213] or affinity based (e.g., frontal affinity chromatography),[138,139,180,214,215] and often use mass spectrometric (MS) detection to aid in compound identification.[216] Applying these methods to mixtures also allows for increased throughput without sacrificing sensitivity or speed of analysis.[217] However, both IMER and affinity-based methods have drawbacks. IMER assays of mixtures require laborious deconvolution by screening each compound in "hit" mixtures individually,[206] while affinity methods require a significant number of control experiments in order to account for compounds that display non-specific binding and provide evidence for the functional relevance of the "hit" ligands.

To overcome these issues, we have developed a new two-stage screening approach that utilizes a functional solid-phase chromatographic assay for primary screening (IMER/MS) followed by an affinity-based method based on biologically-selective solid phase extraction (bioSPE)[143,218,219] coupled to tandem MS to rapidly deconvolute hit mixtures and identify bioactives. A secondary screening method using a different assay format allows for better verification of hits and prevents the occurrence of false positives that may only inflict one assay mode. BioSPE significantly increases throughput of hit

analysis versus the IMER manual deconvolution method. These assays use capillary-scale monolithic silica columns with either an entrapped enzyme (for IMER) or covalently-bound enzyme (to increase binding site density for bioSPE), with the latter column being newly developed for this study. BioSPE has the potential to screen protein targets, such as membrane-bound receptors, that do not have enzymatic functionality and may also be applied to mixtures of increasing complexity and unknown composition using data-dependent MS, such as natural product extracts. We describe optimized methods for performing the combined IMER/bioSPE assay, and demonstrate the rapid screening of synthetic mixtures using adenosine deaminase (ADA) as a model enzyme system.

Adenosine deaminase was chosen as model system in this study, as it builds upon our previous work that used IMER-MS/MS to screen ADA inhibitors,[143] and allows us to demonstrate two key advances over the previous work; a more extensive mixture screen and a new mixture deconvolution method. Adenosine deaminase has become an important drug target due to its role in modulating adenosine concentrations in local tissue environments. Since adenosine concentrations tend to increase in tissues during adverse metabolic changes, the role of ADA is critical for regulating the response of G-protein coupled receptors, and has been implicated in inflammation, myocardial ischemic injury, neurodegenerative disorders and certain types of cancer.[25] There has also been a significant interest in the regulatory role of ADA in the immune system involving the development and function of macrophages, lymphocytes and dendritic cells.[25] The common approach to treating these disorders is by inhibition of ADA to control the

cascade effect of adenosine concentration in downstream signaling events.[25] However, there are few useful spectroscopic assays for assessing ADA activity and inhibition, with the main assays involving UV absorbance, and being difficult to implement in a high-throughput manner. Hence, this enzyme is particularly attractive for development of MS based assay techniques.

Experimental Section

Chemicals. Tetramethylorthosilicate (TMOS, >99%), glycerol, 10 kDa polyethylene glycol (PEG), adenosine deaminase (3.5.4.4 from bovine spleen), 3-aminopropyltriethoxysilane (APTES), ammonium acetate (99.999%), erythro-9-amino-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA, >98%), fluorescein, huperzine A, adenosine (>99%) and glutaraldehyde (8% v/v in H_2O) were obtained from Sigma Aldrich. Diglycerylsilane (DGS) was prepared from glycerol and TMOS as described in detail elsewhere.[197] 250 μm i.d. fused silica capillary tubing was from Cedarlane (Burlington, ON). Bioactive compounds for screening were obtained from the Centre for Microbial Chemical Biology at McMaster University. Distilled deionized water was purified using a Milli-Q A10 Synthesis system. All other reagents were of analytical grade and used as received.

Column Fabrication. Columns were prepared with both entrapped and covalently bound proteins. In both cases DGS was hydrolyzed on ice in a sonicator for 20 minutes with mixing. The sols were allowed to undergo condensation for 40 minutes prior to mixing with the aqueous phase, consisting of either 100 mM HEPES pH 7.0 with 10% (w/v) 10 kDa PEG (blank columns for subsequent protein coupling) or the same buffer containing

44 μM of ADA. Immediately after mixing, these solutions were injected into a 2.5 m length of 250 μm i.d. capillary before phase separation and gelation occurred. Columns were aged for a minimum of 5 days to ensure proper cross-linking of the silica matrix, cut into 10 cm lengths and the 15 cm end pieces were discarded. Details on entrapped column characterization can be found in Supporting Information (SI), Figures S3.1 and S3.2.

For covalent binding of ADA to blank monolithic silica columns, 10 cm segments were first cut from the initial column and washed with 500 μL of a 50% methanol/water solution using a Harvard syringe pump operating at 5 μL/min to remove glycerol and free PEG. The columns were then flushed with 25 μL of ethanol prior to loading 25 μL of 5% (v/v) APTES in ethanol. The reaction was allowed to proceed for 2 hours at ambient temperature followed by flushing the columns with 500 μL of 10 mM HEPES, pH 7.5. 25 μL of a 4% (w/v) solution of gluteraldehyde was then flowed through the column and allowed to react for 2 hours at ambient temperature. Columns were again flushed with 500 μL HEPES buffer to remove any unreacted glutaraldehyde prior to adding the protein.

Adenosine deaminase was buffer exchanged into 10 mM HEPES pH 7.5 using a 10 kDa spin column (Millipore). The columns were loaded with 15 μL of an 84 μM protein solution at 5 μL/min and allowed to react overnight at 4 ºC. The columns were then flushed with 500 μL of the buffer solution, followed by 25 μL of a 500 μM glycine solution to neutralize any unreacted aldehyde functional groups. Details on characterization of covalently bound ADA are described in the SI, Figures S3.3 and S3.4.

Primary Mixture Screen by IMER-MS/MS. 10 cm columns were coupled to an Eksigent 1D-nanoLC system using standard Idex fittings and flushed with 20 mM ammonium acetate, pH 7.5, from 0.5 to 5 μL/min over a 45 minute flow gradient. The columns were equilibrated on the nanoLC at 5 μL/min until the spectrum between 100 and 1500 m/z was stabilized with no visible PEG peaks. Backpressures were consistently between 200 and 300 psi at 5 μL/min for each 10 cm segment used. Columns with entrapped ADA were used for IMER assays and were infused continuously with 25 μM adenosine in 20 mM ammonium acetate with 2% DMSO loaded in channels A and B of the nanoLC. Mixtures were injected using a 5 μL injection loop and hits were determined by analyzing the product:substrate (P/S) ratio via tandem mass spectrometry on a Q-Trap ESI-MS/MS, using 269.2 m/z → 137.1 m/z for inosine (product) and 268.3 m/z → 136.1 m/z for adenosine (substrate), as described previously.[143] Instrument settings are provided in the SI.

Screening was performed on a set of 1080 bioactive compounds using a method similar to that previously reported.[206] Briefly, 54 mixtures were prepared with 20 compounds per mixture at a concentration of 1 μM per compound in 20 mM ammonium acetate, pH 7.5 with 25 μM adenosine and 2% DMSO and placed in an autosampler. The high affinity ligand EHNA was spiked into mixture 53 while a low affinity ligand, MAC-0038732 ((Z)-8-chloro-6-phenylbenzo[f][1,3,5]triazocine-2,4(1H,3H)-diimine, K_d ~ 2 μM),[220] was spiked into mixture 54. To illustrate that the assay was capable of picking up both low and high affinity ligands, mixture 54 was also prepared with compounds at a concentration of 10 μM each and injected using a 20 μL sample loop (54H) using the

same assay conditions as above. This mixture was used to demonstrate that the screen can be biased to detect ligands of varying potency; this biasing method has been previously demonstrated by our group using magnetic bead fishing assays.[221] For each mixture, the product to substrate ratio after infusion of the mixture was compared to that obtained before infusion and converted to a percent activity to generate points on a duplicate plot. Mixtures were considered to be a hit if both duplicates showed less than 50% enzyme activity compared to negative controls.

Mixtures containing a hit were manually deconvoluted using the same protocol for the IMER screen above with each sample containing an individual compound from the hit mixtures. The total amount of DMSO in the mobile phase was reduced to 0.1% DMSO to match the level in the injected samples. For the case of the mixture containing the low affinity ligand, individual compounds were screened using the same conditions as in the low affinity IMER mixture screen. A more rigorous quantification of hits was performed using a competitive IC_{50} assay. The hit compounds were prepared in increasing concentrations spanning the expected IC_{50} in 20 mM ammonium acetate, pH 7.5 continaing 100 μM adenosine. The ADA column was injected with 5 μL of each sample, or 20 μL for the low affinity ligand, to obtain a P/S ratio for each inhibitor concentration. These values were normalized to the P/S ratio in the absence of inhibitor (set to 100%). Zero percent activity was assessed by injecting 100 μM adenosine on a blank silica column. IC_{50} values were obtained from the point where the relative activity decreased to 50% of its initial value using the Hill equation in SigmaPlot 10.0.

Bio-Solid-Phase Extraction Assays. The basic concept of the bioSPE assay is similar to that described for column-based immunoextraction,[143] and is shown in Figure 3.1. When used for deconvolution of "hit" mixtures obtained from IMER, the method involves continuously infusing the column with the bioactive compound mixture until it reaches equilibrium, as determined by a plateau in the total ion current (TIC). Buffer is then infused to remove any unbound compounds from the stationary phase, causing a decrease in the TIC to a lower plateau. Finally, a harsh solvent is infused to denature the immobilized protein and elute any strongly bound ligands, leading to a large peak in the TIC that can be used to trigger data-dependent MS analyses to identify the ligand.

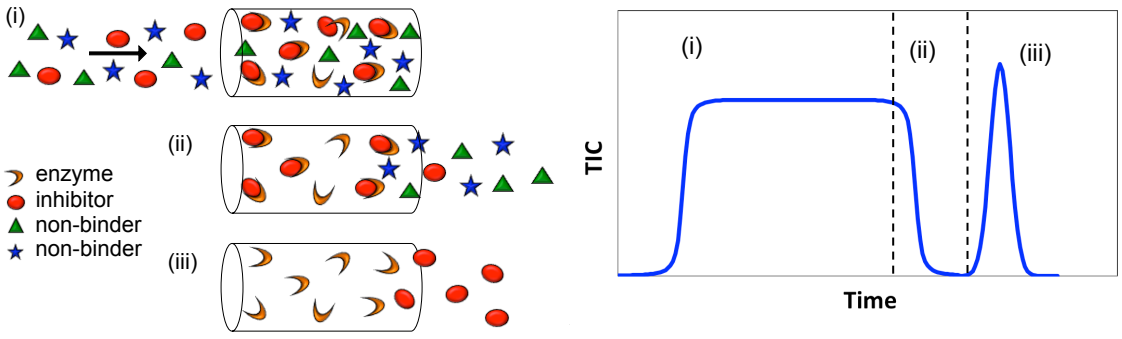

Figure 3.1. Proof of concept for bioSPE using an immobilized enzyme monolithic silica column. (i) Infusion and equilibration of a mixture on the column; inhibitors are preconcentrated on the column by binding to the enzyme while analytes with no interaction with the enzyme will not be retained. (ii) A mild washing containing high ionic strength and MS compatible ammonium acetate is applied to the column to remove any non-specifically bound analytes from the column. (iii) The inhibitor is eluted from the column using a harsh wash, either a denaturant or a competitive ligand.

Initial studies utilized a simple three-component mixture containing EHNA (a potent ADA inhibitor), trimethoprim (void marker) and fluorescein infused into ADA loaded or blank columns. Infusion of a sample containing 1 µM of each compound in 20 mM

ammonium acetate, pH 7.5, was done using an 85 μL injection loop on the autosampler, with each compound monitored separately using multiple reaction monitoring (EHNA: 278.2 m/z → 136.1 m/z, trimethoprim: 291.2 m/z → 230.0 m/z, or fluorescein: 333.3 m/z → 287.1 m/z). Once the signals had plateaued and stabilized, unbound ligands were washed using 20 mM ammonium acetate until the analyte signals had returned to baseline. Elution of EHNA was achieved by injecting either 50% methanol in Milli-Q water, or 3% acetic acid in Milli-Q water. Competitive displacement of EHNA was assessed by injecting 10 μM to 100 μM adenosine onto the column, or 30 μM MAC-0038732. Competitive displacement of 1 μM MAC-0038732 (298.3 m/z → 281.1 m/z) was also tested with 10 μM to 100 μM adenosine and 1 μM EHNA. Elution of the bound inhibitors was gauged based on peak height, width and area relative to blank sol-gel derived silica columns. Column reusability and peak reproducibility were evaluated by performing 8 repeated injections on a single ADA column in one day. The amount of EHNA extracted was quantified using an EHNA calibration curve prepared in 3% acetic acid.

Optimization of ligand extraction from mixtures was carried out using a synthetic mixture of 20 compounds, containing EHNA as a positive control, prepared with 1 μM of each compound in 20 mM ammonium acetate with and without 2% DMSO. MRM transitions for each compound in the mixtures are provided in the SI (Table S3.1). The 20-compound mixture was loaded onto an ADA-loaded silica or a heat denatured ADA column using the autosampler with an 85 μL injection loop at 5 μL/min. Elution from the ADA column was compared to that of blank columns using 3% acetic acid to determine

optimal conditions to specifically and reproducibly retain EHNA versus the other analytes present in the mixture. More complex mixtures required further optimization of the number of column bed volumes (1 bed volume = 5 μL) of 200 mM ammonium acetate used during the wash step to reduce peaks arising from non-specific binding of other analytes in the elution phase.

Mixtures that were identified as having ADA inhibitors were first deconvoluted by running individual IMER assays of each compound (see above) to determine the identity of the bioactive compound, followed by deconvolution of the mixture in a single assay using bioSPE. Mixtures were loaded using an 85 μL injection loop to reach a signal plateau, washed with 18 column volumes of 200 mM NH_4OAc pH 7.5 to remove unbound ligands, followed by addition of 3% acetic acid to produce a bioSPE peak. In the first case all 20 compounds in a given mass encoded mixture were monitored simultaneously via MRM, and the "hit" compound was identified as the one that gave a bioSPE peak. To demonstrate that the assay could also be used with non-encoded mixtures, the MS system was set up to perform data-dependent acquisition (DDA) during the harsh wash step to allow identification of species in the bioSPE peak. The MRM transitions for EHNA (278.2 → 136.1 m/z) and epibatidine (208.9 → 126.1 m/z) were also monitored as controls. Enhanced mass spectra were collected as survey scans across the range of masses for the bioactive compounds (170 → 340 m/z in 0.0420 s) at a scan rate 4000 Da/s during the elution phase. A threshold value was set at 5000 cps with dynamic background subtraction enabled with no former target ions excluded. An ion exclusion list was generated from an extraction performed on a blank column, including

239.4, 217.4, 200.3, 218.3 and 187.1 m/z. Enhanced product ion (EPI) spectra were collected for the three most intense ions in the survey scan using rolling collision energy around 30 V, and monitoring from 50 to 400 m/z. The resulting EPI spectra were used to identify the compounds that were extracted during the assay. Compounds that gave a 10-fold or greater enhancement in signal were considered to be an inhibitor extracted from the immobilized enzyme. Results of both the MRM and DDA bioSPE experiments were compared to the IMER results.

Results and Discussion

Primary Mixture Screen and IC_{50} of Hit Compounds by IMER-MS/MS. The initial screening of 1080 compounds was performed in duplicate by IMER-MS/MS, with the data for the two assays shown in a duplicate plot in Figure 3.2A. This screen identified mixtures 7, 20, 53 (EHNA spike) and 54H (MAC-0038732 low affinity bias) as containing compounds that reduced the P/S ratio below the 50% value we considered to be a hit. Mixtures 7, 20 and 53 in fact showed a drop in relative activity to less than 15%. Mixtures 7, 20 and 54H were then manually deconvoluted by running IMER-MS/MS assays on each compound in the mixtures to determine the compound responsible for inhibition (Figures 3.2B-D). Mixture 54 was not manually deconvoluted since the "hit" compound, EHNA, was already known, IMER based deconvolution of EHNA has already been demonstrated,[143] and because it was intended to be used as a positive control in bioSPE-based deconvolution assays. The hit compound in mixture 7 was determined to be vidarabine (9-β-D-arabinofuranosyladenine). However, this compound is not truly an

inhibitor, but a competing substrate that turns over at a slower rate than adenosine, thus competitively inhibiting adenosine turnover.[222] The hit compound from mixture 20 was determined to be tubercidin. However, this compound was determined to be a false positive since its ^{13}C isotope interferes with the adenosine MRM signal at 268.3 → 136.1 m/z, causing a decrease in the P/S ratio. Mixture 54, with 1 μM of MAC-0038732, was initially detected outside the hit window at ~83% activity, showing a minimal reduction in ADA activity. When using mixture 54H with 10 μM of each compound to bias the assay to low-affinity ligands, MAC-0038732 was detected within the upper left quadrant of the 50% hit window. Interestingly, this compound took longer to produce a decrease in product signal and increase in substrate signal, as it required a higher concentration and a larger volume to produce a signal change, and likely indicates a slow on-rate to the enzyme active site versus vidarabine and EHNA. Overall, the manual mixture deconvolution and analysis of individual components of one mixture required about 7 hours to perform. Despite the low hit ratio in this screen of ~0.3%, this is still a significant amount of time to invest in manual deconvolution and demonstrates the need for a higher throughput method of mixture deconvolution.

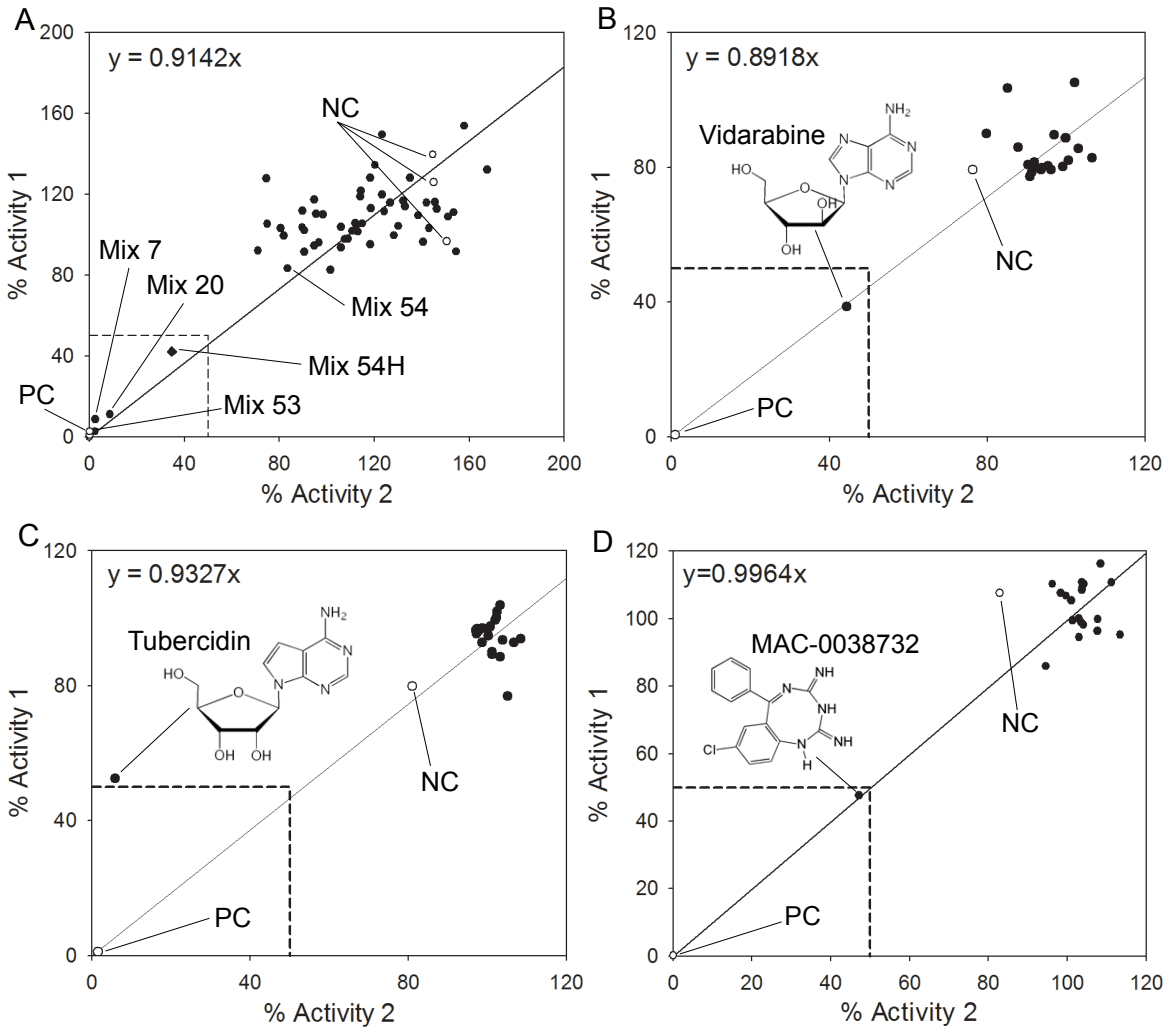

Figure 3.2. (A) ADA IMER duplicate plots showing (●) mixtures, positive (PC, ○), negative controls (NC, ○) and low affinity biased mixture (◆). Mixtures 7 and 20 were identified as hits. (B) Deconvolution screen of mixture 7 identified vidarabine as the hit compound. (C) Mixture 20 identified tubercidin, a false positive based on the ^{13}C isotope of tubercidin (268 → 136 m/z) falsely increasing the substrate concentration and therefore reducing P/S, resulting in a 'hit' identification. (D) Mixture 54 containing a low affinity inhibitor was only detected when the screening parameters were biased with a larger detection volume and higher inhibitor concentration.

The potency of the hit compounds was assessed by performing on-column IMER-based IC_{50} assays. The hits in mixtures 7 and 20 are structurally similar to adenosine and

add an extra complication to IMER analysis by having interfering MRM transitions for the monoisotopic $[M+H]^+$ peak and/or ^{13}C isotope peaks. Corrections were made to percent activity by running the assay on the ADA column in the absence of adenosine and subtracting the areas of the interfering peaks. The adenosine concentration used to generate IC_{50} curves was 100 μM, since higher concentrations were required for the hit compounds in order to maintain pseudo first order reaction kinetics. Figure 3.3 shows the IC_{50} curves for EHNA, vidarabine, tubercidin and MAC-0038732. EHNA had an IC_{50} of 6 ± 1 nM, corresponding to a K_I of 1 nM, which agrees well with our previous report.[143] Vidarabine, although not a true inhibitor, could still decrease the activity of the entrapped enzyme, resulting in an apparent IC_{50} of 358 ± 2 nM, though this was due to the compound being a competitive substrate with a slow turnover rather than a true inhibitor. The low affinity inhibitor MAC-0038732 had an IC_{50} of 17.2 ± 0.2 μM, corresponding to a K_I of 3.43 ± 0.05 μM, which is in good agreement of the previously reported value of 2 μM.[220] Tubercidin showed no inhibition of ADA (relatively flat IC_{50} curve), confirming that this compound was a false positive.

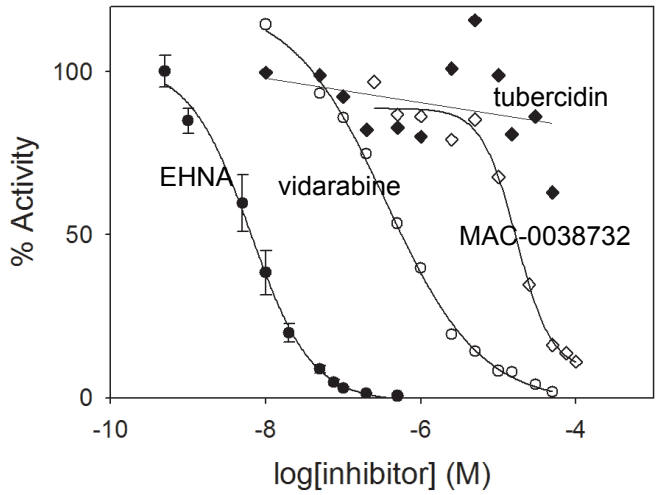

Figure 3.3. IC_{50} curves for EHNA (●) 6±1 nM, vidarabine (○) 358 nM, MAC-0038732 (◇) 17 µM, and tubercidin (◆), which showed no inhibitory activity upon signal correction. The IC_{50} for EHNA was performed in triplicate while the other components were of limited quantity from the compound library and were only performed in one assay since duplicate runs were necessary to correct for adenosine signal overlap with the analyte ^{13}C isotopes.

BioSPE Column Optimization. Rapid isolation and identification of inhibitors from a hit mixtures identified by IMER, using an automated chromatographic assay, was the primary goal of developing bioSPE. This assay will significantly decrease the amount of time to identify hit compounds within a complex mixture. Columns were initially tested for their ability to perform an enzyme-specific bioextraction of inhibitors from hit mixtures. Optimization included initial infusion volume, buffer type and buffer wash volume, and the nature of the elution solvent, with the height and area of the elution peak for an active column relative to a blank (as judged via its MRM transition) being the output signal. Selectivity of extraction was also determined by assessing the amount of inhibitor relative to other analytes eluting within the harsh washing phase.

To provide a large number of binding sites, columns were prepared by the covalent immobilization method for bioSPE assay development. The protein loading was much greater using this method versus sol-gel entrapment and could be quantified using FAC (Figure S3.3) resulting in 712 ± 17 pmol ADA per column, a 500-fold increase in protein loading versus the entrapped ADA columns (1.42 ± 0.04 pmol). This lead to a significant increase in column activity and hence the amount of ligand that could be effectively isolated and extracted from mixtures, as demonstrated below. Initially, bioSPE was optimized with covalently bound ADA columns using a simple 3-component mixture containing 10 μM each of EHNA, fluorescein and trimethoprim. Loading of the mixture was performed until the TIC came to a plateau, indicating that the analytes had reached equilibrium with the stationary phase. A frontal affinity shift could be observed for compounds that exhibited either affinity for the immobilized enzyme or non-specific binding to the stationary phase (Figure 3.4). On a glycine-functionalized column, there was no observed retention of EHNA, while on an ADA-immobilized column the EHNA signal was shifted to longer retention times prior to coming to equilibrium, indicating binding to ADA. Fluorescein, which was originally selected as a void marker owing to its lack of interaction with anionic silica stationary phases,[214] was non-specifically retained in both blank and ADA derivatized columns, indicating that the charge of the stationary phase was reversed, likely owing to excess amine groups that were not coupled to gluteraldehyde. Trimethophrim showed no retention on either blank or ADA loaded columns, and hence this compound was chosen as the void marker, while fluorescein was used to assess the washing of non-specific binders from the column.

Upon reaching a signal plateau, washing of column to elute loosely bound or non-specifically bound compounds was evaluated using both 20 mM and 200 mM ammonium acetate. The higher ionic strength buffer allowed for a smaller wash volume to remove loosely bound compounds while giving better retention of specifically bound analytes. Figures 3.4A and 3.4B illustrate the facile removal of non-specifically bound fluorescein prior to the elution stage using a 200 mM ammonium acetate wash. Despite having a much larger frontal retention shift versus EHNA, the mild wash phase was successful in removing fluorescein in as little as 8 column bed volumes. For mixtures of higher complexity, this volume had to be increased to 18 bed volumes (see below).

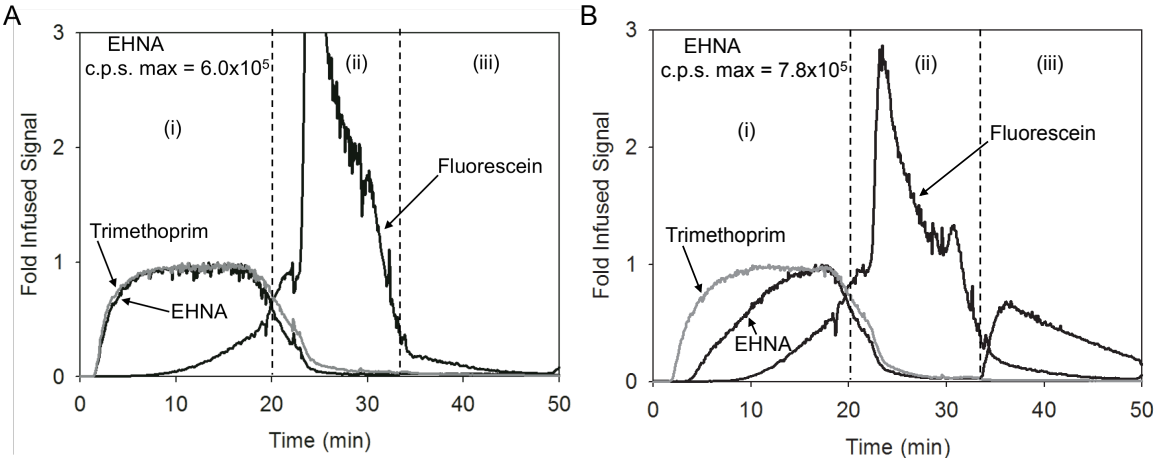

Figure 3.4. BioSPE optimization using (A) a glycine functionalized column and (B) an ADA functionalized column. Both columns were (i) infused with a 10 µM analyte mixture, (ii) washed with 200 mM buffer at 20 minutes and (iii) eluted with 50% methanol at 33 minutes. The glycine column shows no retention of EHNA during either the infusion or elution phase whereas the ADA column shows both a frontal shift and a bioSPE peak.

Elution of EHNA was tested with 50% methanol and 3% acetic acid on an ADA column (Figure 3.5A). The loading concentration was set at 1 µM to match the initial

concentration used in the primary IMER screen. Improved peak resolution and greater signal intensity were observed using the 3% acetic acid elution as compared to 50% MeOH. Elution from a column with entrapped ADA is also shown to demonstrate the immense improvement in signal levels obtained with the covalently immobilized ADA versus entrapped ADA when performing bioSPE.

To demonstrate that bioSPE could work without denaturing the immobilized protein and identify active site specificity of the bound inhibitor, a series of competitive displacement assays were performed using a 1 μM EHNA solution was and a harsh wash containing either adenosine or MAC-0038732 (Figure 3.5B). The lower affinity ligands did not result in a significant increase in EHNA signal or produce any resolvable bioSPE peak. However, when 10 μM MAC-0038732 was loaded on the ADA column and eluted with a 1 μM solution of the more potent EHNA inhibitor, a resolved and intense peak was observed. Thus low affinity ligands can be eluted with potent ligands, while weaker affinity ligands are not able to displace high affinity ligands in a well-resolved manner. In cases where a potent ligand is not available for competitive elution in bioSPE, our previously reported competitive displacement chromatography method may be used, as this method can utilize weakly binding ligands to partially displace a more potent inhibitor from a column in sufficient quantity to allow identification by mass spectrometry.[8]

To ensure ligands were binding specifically to native ADA, a column was prepared with heat denatured ADA and binding of EHNA was examined on both columns using the optimized assay parameters. Figure 3.5C shows that EHNA produced a bioSPE peak

only for native ADA columns, ruling out non-specific binding to ADA, and confirming a functional interaction with native ADA. The control compound trimethoprim did not display a bioSPE peak in either column (data not shown), further confirming the selectivity of the EHNA-ADA interaction. Overall, when using the optimized load/wash/elute procedure, a 23-fold increase in signal intensity over the infused signal maximum was observed for EHNA upon extraction from the ADA column using 3% acetic acid. The shape of the elution peak is affected by the increased ionization efficiency of EHNA in acetic acid and results in a tailing peak while the inhibitor is being slowly removed from the immobilized enzyme. The MRM signal does not return to baseline until the elution phase is complete and the injection loop is emptied of acetic acid, which causes a loss of ionization and a rapid drop at the end of the elution peak. Future assays will aim to shorten the elution time in order to reduce this effect. After the full sample loop of acetic acid is flowed through the column, the inhibitor is completely removed from the column. A second injection of acetic acid without loading the inhibitor on the column does not produce an elution peak, only an increase in ionization efficiency below the infusion signal maximum, indicating that carry-over between runs is not occurring.

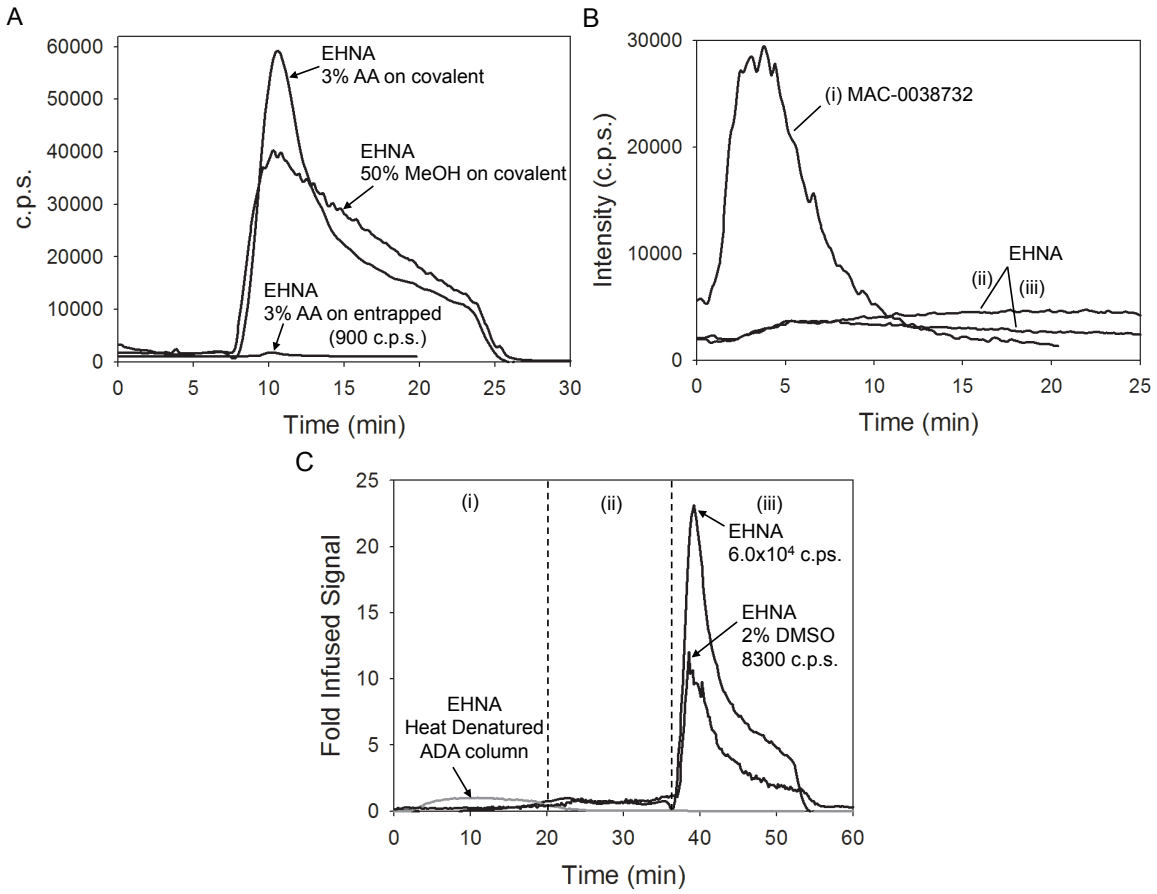

Figure 3.5. (A) Comparison of EHNA extraction on covalent and entrapped ADA columns using 1 µM simple mixture. (B) Comparison of competitive displacers on ADA column during (i) elution of MAC-0038732 using 1 µM EHNA, (ii) EHNA using 30 µM MAC-0038732, and (iii) elution of EHNA using 100 µM adenosine. (C) Comparison of ADA and heat denatured ADA columns during (i) infusion of 1 µM EHNA mixture with or without 2% DMSO, (ii) wash with 200 mM buffer at 20 minutes and (iii) extraction at 37 minutes using 3% acetic acid. Extraction is specific to active ADA column with reduced signal intensity when mix is infused in the presence of DMSO.

As with many compound libraries, the bioactives used in this screen were stored in DMSO. It is well known that DMSO forms adducts during ESI-MS, causing signal attenuation.[223] To determine if the bioSPE assay produced an adequate signal in the presence of DMSO, a sample spiked with 1µM EHNA was prepared in the presence and

absence of DMSO. MRM transitions were optimized for each sample (Table S3.1) and monitored throughout the bioSPE process. The extracted EHNA peak when the assay was performed in the presence of DMSO was 7 times smaller compared to performing the assay in aqueous conditions (Figure 3.5C). Since much of the DMSO was removed during the mild wash, a 12-fold signal enhancement over the infused signal maximum was still achieved. Thus, bioSPE is capable of a significant signal enhancement, even in the presence of 2% DMSO.

Ligand Extraction from Mixtures. Hit mixutres 7, 20, 53 and 54H were prepared in the absence of DMSO for rapid compound identification via bioSPE. DMSO was not used so as to give the best signal enhancement possible during the assay. Each compound was monitored by MRM to monitor infusion of all compounds in the mixture and evaluate their interference. During the mixture infusion onto the column (Figure 3.6A), EHNA had a shift in frontal elution while the remainder of the compounds had a very similar frontal shift, eluting with the void volume or just slightly after. Compounds that had shifts in retention were effectively washed from the column using the 200 mM buffer wash prior to elution. None of the compounds had any observed bioSPE signal enhancement during the elution step as compared to the infusion signal except EHNA, which had a 23-fold increase in signal compared to initial loading. Replicate extractions of EHNA from the mixture on a single column were reproducible with an RSD of 8.8% (See SI Figure S3.4). Quantification using an EHNA calibration curve determined that 140 ± 10 pmol was extracted from the column, indicating that approximately 15% of the ADA binding sites on the column were occupied by EHNA after the loading and washing

steps. An important point is that the bioSPE mixture deconvolution required only 40 – 60 minutes compared to > 7 hours for manual deconvolution of individual compounds by IMER. This is a significant increase in throughput while also reducing the amount of sample preparation.

The low affinity inhibitor MAC-0038732 was also extracted from an aqueous 20 compound mixture (Figure 3.6B). The signal enhancement from the eluted peak was 52-fold greater than the infused signal. However, the peak area and width was also much smaller, indicative of a lower affinity ligand that is more easily removed from the immobilized enzyme active site. Application of the hit mixtures containing vidarabine and tubercidin to the ADA column did not produce a bioSPE peak during the elultion step. Tubercidin showed no peak, while vidarabine underwent turnover on the column and was detected as 9-β-D-arabinofuranosylhypoxanthine. Hence, in both of these primary "hit" mixtures, the bioSPE assay was capable of discerning hits from false positives in the IMER screen.

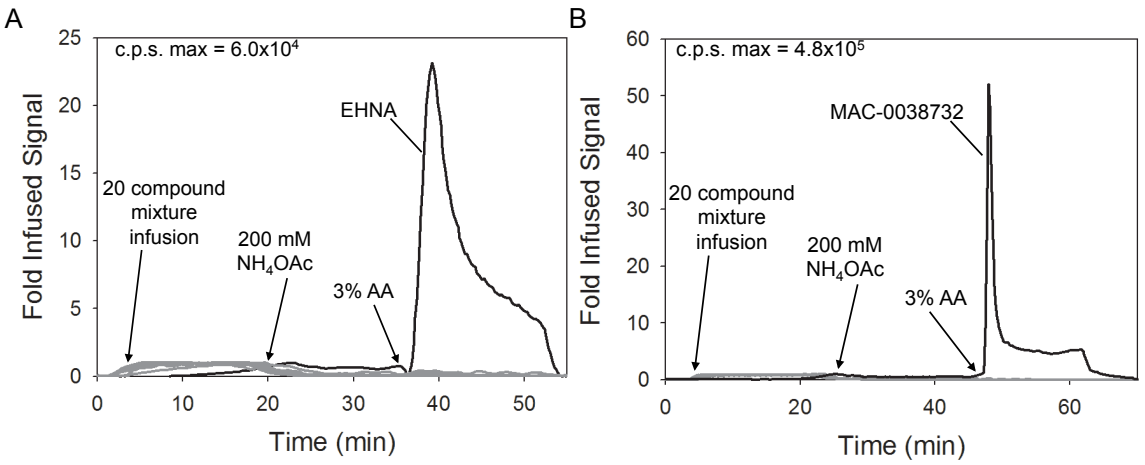

Figure 3.6. (A) Infusion of a 20 compound mixture spiked with EHNA, each compound at 1 µM, monitored by individual MRM transitions. A frontal affinity shift of EHNA can be observed upon infusion of the mixture. Elution with 3% acetic acid shows a 23-fold increase in signal. (B) BioSPE of low affinity MAC-0038732 from 20 compound mixture shows a 52-fold increase in signal.

Data-Dependent BioSPE. Although MRM mode is beneficial for mass encoded libraries when monitoring specific compound-protein interactions and during assay development, optimizing MRM transitions is time consuming and there are a limited number of transitions that can be monitored simultaneously. There are also situations where the mixture components are not known, such as natural product extracts. In such a case, data-dependent acquisition (DDA) can be used to trigger the MS to collect enhanced product ion (EPI) spectra when the TIC moves above a pre-defined level during the elution step. To demonstrate the utility of bioSPE to non-mass encoded mixtures, DDA-MS was performed on both mixtures 53 and 54H at the start of the elution phase with 3% acetic acid to identify compounds eluting from the ADA column (Figure 3.7). Both EHNA and MAC-0038732 were exclusively detected from their 20 compound mixtures as an

increase in $[M+H]^+$ ions during the enhanced mass spectrum (EMS) survey scan at 278.3 m/z and 298.2 m/z respectively. The rise of these ions triggered EPI spectra to be collected, generating EHNA and MAC-0038732 fragmentation patterns that matched the anticipated spectrum of the inhibitors. The signal enhancement for the EHNA elution peak, as detected in the XIC of the 278 m/z ion was 12-fold greater than infused signal, while for MAC-0038732 the signal enhancement was 10-fold greater. After the elution peak hits a maximum, ions detected by DDA are not representative of ions being eluted from the column since DDA is set to detect ions that are rising from the background only and produce XICs with no signal enhancement during the elution phase. This makes differentiation between DDA detected inhibitors and noise as simple as viewing the XICs of selected ions and evaluating whether a peak is present with approximately 10-fold or greater signal enhancement. The spectral data obtained by DDA-MS bioSPE requires a more vigorous analysis to identify the inhibitor, but also saves a significant amount of time in sample preparation, and assay optimization and can be applied to complex mixtures that are not mass encoded.

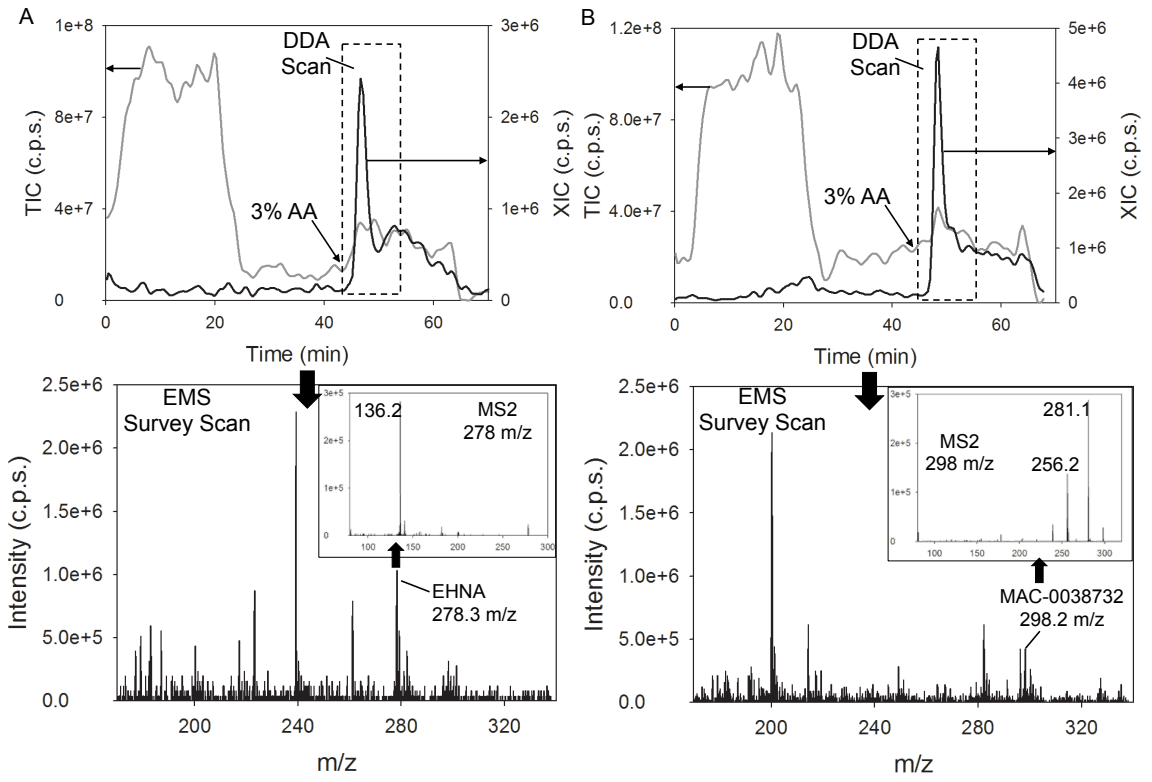

Figure 3.7. Information dependent acquisition of extracted EHNA from ADA column. (A) TIC (—) overlayed with EHNA (—). Infusion signal of EHNA is not detected in EMS while extraction results in a peak 10 times greater than baseline. Only EHNA (278.4 m/z) was detected in the survey scan during elution. (B) EMS survey scan during extraction. Peak reject list excludes ions present from background contamination. (C) EPI spectrum collected for EHNA during the peak elution shows the expected fragmentation pattern.

Conclusions

Bio-solid phase extraction of compounds from complex mixtures has proven to be a simple and rapid method to isolate and identify inhibitors of adenosine deaminase. This study is the first instance of an enzymatic stationary phase being used to isolate and extract inhibitors from complex mixtures for drug discovery assay development.

Immobilized enzyme columns can be fabricated with high protein loading using a simple covalent linkage reaction to increase ligand-binding capacity while improving assay robustness and reproducibility. When used in conjunction with IMER, bioSPE becomes a powerful secondary method to confirm and identify hits that have already been found to target enzyme function. Two compounds, EHNA (potent ligand) and MAC-0038732 (weak ligand), were successfully isolated and identified by bioSPE both in MRM and DDA-MS modes. The assay is rapid and easy to perform on mixtures even when the mixture is infused in the presence of DMSO. To further increase throughput and reduce interference from analyte signals, columns may be loaded and washed offline provided the wash phase has been optimized to prevent non-specific binding to the stationary phase. The elution phase can either use a denaturing solvent to separate the enzyme-inhibitor complex, or if a potent ligand is already known it can be used to compete the analyte from the active site. BioSPE also has the potential be applied to non-enzymatic systems to identify protein-binding ligands since it does not require the turnover of a substrate to produce a signal and could be used as a primary screening method for these systems using data-dependent acquisition for identification of unknown ligands.

CHAPTER 3. SUPPLEMENTARY INFORMATION

Experimental Data

Column Leaching. Eluate from 10 cm column segments were collected in 15 µL fractions, mixed with a 10 µL aliquot of 100 µM adenosine and allowed to react for 5 minutes. The reaction was quenched with 75 µL of methanol followed by injection into an AB Sciex QTrap API 2000. The resulting solutions were analyzed in multiple reaction monitoring (MRM) mode for adenosine (268 → 136 m/z) and inosine (269 → 137 m/z) signal ratios and compared to a calibration curve to determine the amount of enzyme leached from the column.

Column Characterization by Michaelis Menten Kinetics. Column activity was assessed on entrapped ADA columns by injecting increasing concentrations of adenosine up to 500 µM via an Eksigent AS-1 autosampler coupled to the ADA column and then connected to the ESI source. The flow rate was 5 µL/min and was teed prior to the source to a makeup flow of 1% acetic acid in LCMS grade methanol at a rate of 5 µL/min. Calibration curves were prepared via a previously described method[143] to correct for the ^{13}C isotope of adenosine interfering with the inosine MRM transition.

Column Characterization by bioSPE. Entrapped ADA columns were used to determine the protein loading by infusing EHNA at increasing concentrations from 10 nM to 2 µM. The columns were washed with 8 bed volumes of 20 mM ammonium acetate prior to elution with 3% acetic acid. The resulting peaks were quantified by use of an EHNA calibration curve prepared in 3% acetic acid. Extracted peaks were plotted versus the

infused concentration to determine the maximal loaded concentration and dissociation constants by fitting data to one-site saturation ligand binding using SigmaPlot 10.0.

Mass Spectrometer Settings. Instrument settings for IMER assays were as follow: curtain gas = 45.0, collision gas = medium, ion spray voltage = 5500V, temperature = 200 °C, declustering potential = 45 V, exit potential = 11 V, collision energy = 26 V, cell exit potential = 3.0 for both the adenosine and inosine MRM transitions. Conditions for compounds used in bioSPE assays are provided in Table S3.1.

Table S3.1. MRM transitions for bioactive compounds in screening mixture

Compound ID	Q1	Q3	Time (msec)	DP (V)	EP (V)	CE (V)	CXP (V)
N5-butyl-1,2,4-thiadiazole-3,5-diamine	173.2	117.1	500	30	10	30	3
N'-(2,6-dimethoxybenzoyl) nicotinohydrazide	179.2	90	500	68	10	41	2.5
3,8-dithia-1,6-diazaspiro[4.4]nona-1,6-diene-2,7-diamine	189.1	113.1	500	39	10	21	3
1-[(3-pyridylamino)methyl] pyrrolidine-2,5-dione	206.2	107	500	30	10	30	3
epibatidine	208.9	126.1	500	62	11	32	3
1-{[(6-methyl-2-pyridyl)amino]methyl} pyrrolidine-2,5-dione	220.1	121.2	500	31	8	23	3
2-[2-(2-propyn-1-ylsulfanyl)phenyl]-1,4,5,6-tetrahydropyrimidine	231.2	192.2	500	45	10	27	3
huperzine A	243.2	226.2	500	62	11	30	4
N2-[3-(1H-imidazol-1-yl)propyl]-3-nitropyridin-2-amine	248.2	180.2	500	35	9	20	3
pyrimethamine	249.2	177.1	500	83	11	39	4
N-{2-[[(acetoxy)imino](amino) methyl]-3-fluorophenyl}acetamide	254.2	152.2	500	32	9	16	3

vidarabine	268.2	136.1	500	65	10	30	3
methyl-N-(4-methoxyphenyl)-4-morpholinecarimidothioate	267.2	180.1	500	45	8	27	3
tubercidin	267.2	135.1	500	50	11	27	3
2-(4-chlorophenyl)-2-oxoethyl-N,N-dimethylcarbamodithioate	274.1	88.1	500	27	8	28	3
sanguinine	274.3	199	500	65	11	32	3
(erythro-9-(2-hydroxy-3-nonyl)adenine)	278.2	136.1	500	68	10	30	3
galanthamine	288	213.1	500	65	11	31	3
trimethoprim	291.2	230	500	80	10	33	3
N'-(2,6-dimethoxybenzoyl)nicotinohydrazide	302.2	165.2	500	30	10	30	3
N-[2-(diethylamino)ethyl]-2,3,4,5,6-pentamethylbenzenesulfonamide	327.3	100.2	500	60	11	31	2
6-{[3-(dimethylamino)propyl]amino}-2-morpholino-3-nitrobenzonitrile	334.3	230.2	500	42	10	27	3.5

Entrapped ADA Column Optimization. Sol-gel entrapped ADA columns were tested with a simple mixture containing EHNA, fluorescein and huperzine A at concentrations ranging from 10 nM to 2 μM. Mixtures were loaded onto the column using an 85 μL injection loop, washed with 200 mM ammonium acetate pH 7.5, then eluted with either 50% methanol or 3% acetic acid. Competitive displacement of EHNA was also assessed using either 25 μM or 100 μM adenosine.

Column Characterization by FAC. Protein binding sites (B_T in picomoles) were quantified using frontal affinity chromatography-tandem mass spectrometry[138] (FAC-MS/MS) by running increasing concentrations of EHNA, from 1 to 10 μM, through a

series of columns (using a fresh column for each ligand concentration) and fitting the data to Equation (1):

$$V - V_o = \frac{B_T}{[A] + K_d} \quad (1)$$

where V_0 is the void volume (μL), V is the retention volume (μL), [A] is the concentration of EHNA (μM), and K_D is the binding constant of the ligand to the protein (μM). The retention volume was determined as the volume where the frontal curve reached 50% of the maximum intensity. Columns with entrapped protein had too small a protein concentration to provide observable shifts in elution volume relative to the void volume, and instead had protein loading calculated by measuring the relative turnover of adenosine to inosine as compared to columns with covalently bound proteins.

The covalently bound ADA columns were assessed for reproducibility by performing replicate extractions of EHNA on a single ADA column. Columns were loaded with 85 μL of a simple ternary mixture containing 1 μM each of EHNA, fluorescein and trimethoprim, then washed with 200 mM ammonium acetate pH 7.5 for 10 column bed volumes, followed by elution with 3% acetic acid. A calibration curve was generated using EHNA in 3% acetic acid from 50 nM to 10 μM in order to quantify the amount of EHNA extracted from the column. Replicate injections of the mixture were assessed for reproducibility by measuring the EHNA XIC area extracted from the same column.

Results and Discussion

Column Leaching and Activity. Leaching of the enzyme from the column was observed for the first 8 bed volumes when flushing at 5 μL/min (Figure S3.1A). The amount of enzyme lost on each column is negligible after this volume and was therefore used as a minimum conditioning procedure prior to using. Without adequate conditioning, PEG and glycerol appear to suppress enzyme function, which is partially due to the microviscosity they impart in the silica matrix that interferes with the pressure driven diffusion of analytes into the mesopores and hence contact with the enzyme, as well as interfering with MS detection by causing ion suppression of analytes of interest. Once conditioned, the Michealis-Menten constant was determined to be 20 ± 7 μM (Figure S3.1B). This is within error of the solution value of 24 μM[224] and a higher affinity compared to our previous report on ADA activity on column of 100 μM.[143] Enzyme reactor mode is a beneficial way of running initial kinetic studies of the target biomolecule since it does not subject it to a harsh wash and can be used repeatedly for hundreds of injections without inhibiting function.[206]

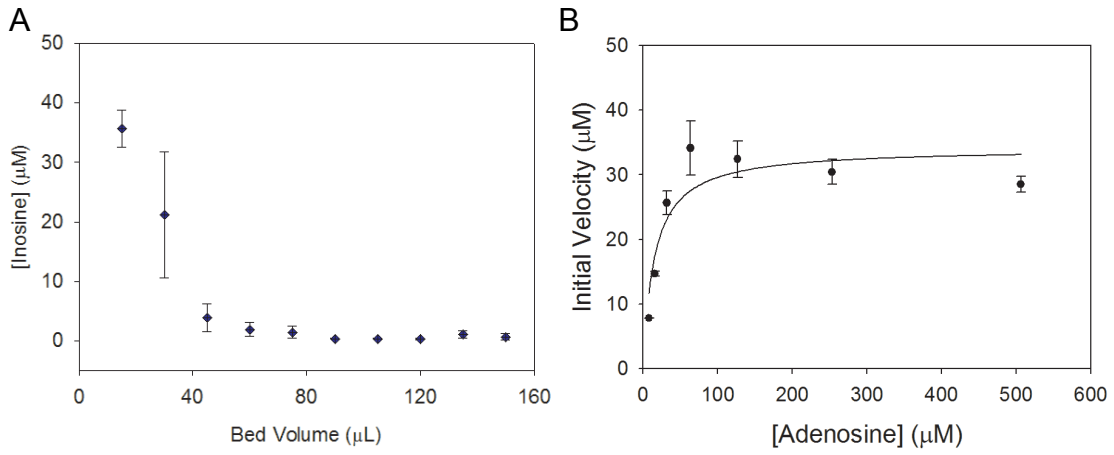

Figure S3.1. (A) Leaching of adenosine deaminase from a 10 cm sol-gel entrapped ADA column. Eight bed volumes are sufficient for removing leachable protein as a way of pre-conditioning the column prior to use. (B) Enzyme activity versus adenosine concentration on entrapped ADA columns shows a K_M value of 20 ± 7 μM, as determined by fitting the data to a Lineweaver-Burke model.

Entrapped ADA Column Optimization. ADA columns produced via sol-gel entrapment of the enzyme were tested for their ability to extract EHNA from a simple mixture containing EHNA, fluorescein and huperzine A. Figure S3.2A shows that both 50% methanol and 3% acetic acid were capable of effectively separating EHNA from fluorescein and huperzine A. A competitive extraction assay was also performed using adenosine as the elution solvent to confirm the presence of an active site inhibitor versus an allosteric inhibitor (Figure S3.2B). An increase in substrate concentration during elution shows an increase in EHNA signal. However, adenosine does not provide a signal enhancement of EHNA as with 50% methanol or 3% acetic acid. Ion suppression from the high substrate concentrations used coupled with the slow off-rate of EHNA contributes to reduced signal enhancement and elongated peak widths. Successive extractions could not be performed on the entrapped ADA columns without a significant

loss of activity, eventually leading to a complete loss of activity after 4 repeated extractions. Once mixture complexity was increased on the columns, the separation efficiency of the columns decreased, leading to incomplete extraction of EHNA versus non-specific binders. Initial bioSPE proof of concept and optimization studies using columns with entrapped ADA did not provide a reproducible elution peak during the harsh washing step, which was determined to be the result of inadequate protein loading. All subsequent bioSPE assays used columns with covalently bound ADA, which produced a much higher number of binding sites (see Results section). Unfortunately, when the entrapped ADA columns were tested with a 20-component EHNA spiked mixture, an extremely low amount of EHNA was extracted. The reason for this was found to be that the amount of protein in the column was only 1.42 ± 0.04 pmol, based on peak areas for EHNA extracted from entrapped ADA columns using 3% acetic acid as compared to the peak area obtained from a column with covalently bound ADA (see Figure S3.2 and compare to Figure 3.5). In addition, protein leaching from the macroporous silica matrix resulted in a continually decreasing and irreproducible protein concentration on the columns. This effect, coupled with interfering signals from PEG and glycerol byproducts remaining from column fabrication, caused a significant decrease in inhibitor retention and increased detection limits, making both frontal affinity chromatography (FAC) and bioSPE unfeasible using entrapped ADA columns.

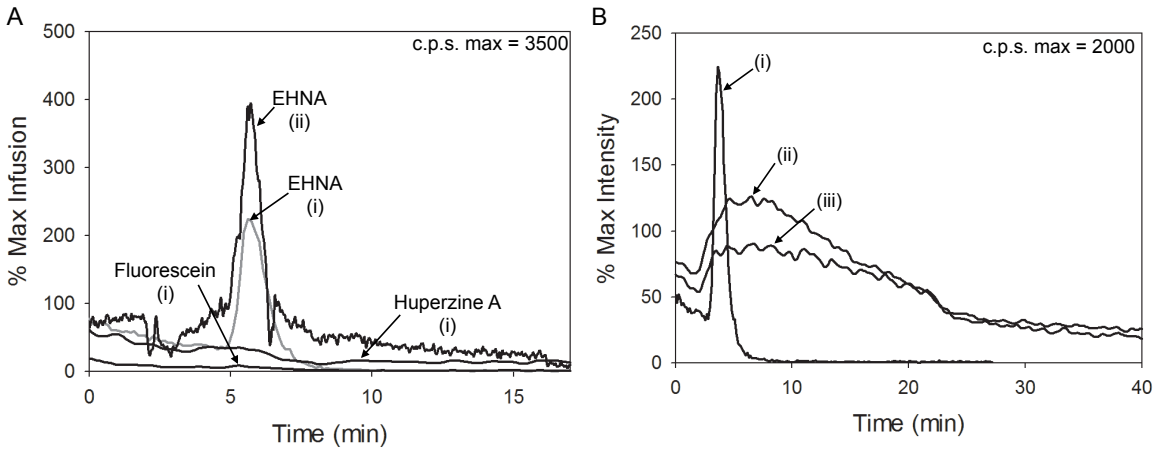

Figure S3.2. (A) Extraction of simple mixture from sol-gel entrapped ADA columns using (i) 50% methanol, and (ii) 3% acetic acid. (B) Extraction of EHNA from a sol-gel entrapped ADA column with (i) denaturing 50% methanol, or competitively with (ii) 25 μM adenosine, and (iii) 100 μM adenosine.

Covalent Column Characterization by Frontal Affinity Chromatography (FAC). The protein loading (B_T) of covalently-bound ADA columns was assessed by FAC using EHNA infused as concentrations ranging from 1 to 10 μM. In cases where the signal could not reach a 100% signal intensity compared to infusion on a heat denatured ADA column, the maximum signal intensity on the heat denatured column was used as 100% signal intensity to calculate the percent infusion for the signal on the functional ADA column. The retention volume at 50% infusion of EHNA on the ADA column was plotted versus EHNA concentration and fitted to equation (1) using Sigma Plot 10.0 software. The column protein loading was characterized via FAC since specific activity was extremely high and the maximum turnover velocity (V_{max}) could not be reached prior to running into ESI-MS ion suppression effects, thus leading to difficulties in determining K_M. An observed 25 minute frontal retention for 10 μM EHNA was used as a starting

point for determining B_T and K_D by FAC. Figure S3.3A shows increased retention volumes with decreasing EHNA concentration and when fitting the data to equation 1 in Figure S3.3B, the protein loading (B_T) was determined to be 712 ± 17 pmol. The inhibitor dissociation constant (K_D) was not reliably determined by the FAC method (25 ± 27 nM), as the amount of functional protein was very high compared to the actual K_D, leading to relatively high error upon curve fitting. Covalent columns were therefore chosen to perform all further bioSPE experiments since they were better at producing higher signal with less interferences with a higher reproducibility.

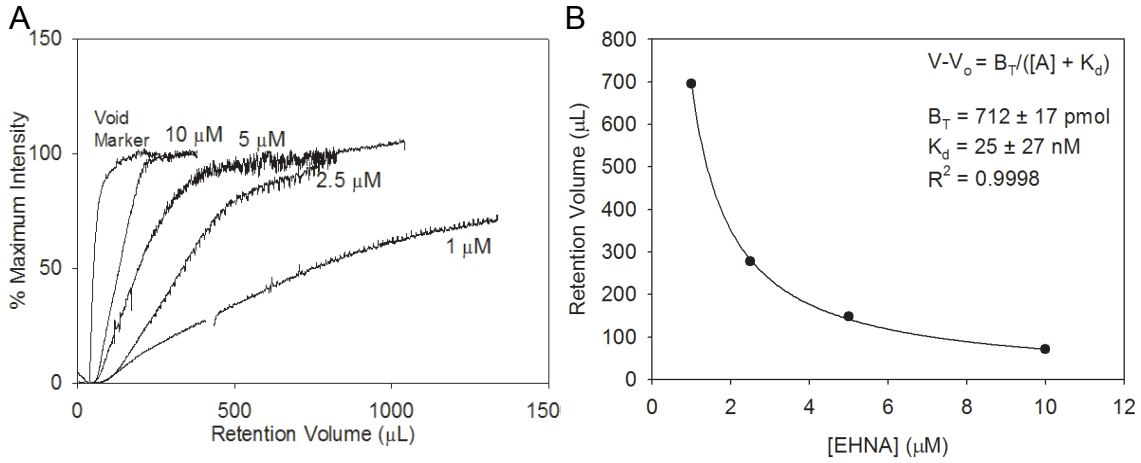

Figure S3.3. Characterization of covalently bound adenosine deaminase monolithic silica columns using separate 10 cm column segments via frontal affinity analysis. Panel (A) depicts the concentration dependent frontal elution time of EHNA compared to the void marker trimethoprim. Panel (B) shows the fit of the FAC equation to the data showing a protein loading (B_T) of 712 ± 17 pmol and a K_D of 25 ± 27 nM. The high standard error could be reduced by performing replicate runs using smaller column segments.

Column Reproducibility. Figure S3.4 shows the reproducibility of EHNA extraction from covalently bound ADA columns. The RSD was 8.8% for 8 replicate extractions with no significant loss in extracted EHNA area over the day tested. This indicates that columns can be reused multiple times for extraction without adversely affecting ADA activity.

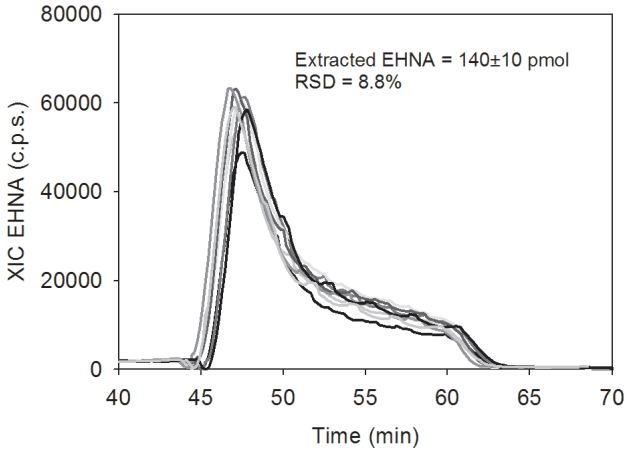

Figure S3.4. Replicate extractions of EHNA from covalently bound ADA column using 3% acetic acid.

CHAPTER 4. SECONDARY METABOLITE EXTRACT SCREENING VIA BIO-SELECTIVE SOLID-PHASE EXTRACTION DATA DEPENDENT TANDEM MASS SPECTROMETRY

This study is targeted for submission to the journal *Analytical Chemistry*:

Forsberg, E.M., Ibrahim, A., Kapteyn, E., Brennan, J.D., **Secondary Metabolite Extract Screening via Bio-Selective Solid-Phase Extraction and Data Dependent Tandem Mass Spectrometry**, (to be submitted July, **2015**).

The author optimized the column fabrication of sol-gel derived monolithic silica, the amine surface modification, gluteraldehyde modification and enzyme coupling reactions. Emily Kapteyn prepared a portion of the ADA columns for the screening assays. The HTS facility provided the aqueous compound library and, in conjunction with the Biointerfaces Institute, prepared the mixtures up to 1000 compounds spiked with the known inhibitors. Asharaf Ibrahim prepared the fungal endophyte extracts for the natural product screen versus the ADA columns, discussed data analysis and further purified hit compounds. Dr. John Brennan and the author drafted the final manuscript.

Abstract

Bio-selective solid-phase extraction (bioSPE) has been previously demonstrated as a method for isolating and identifying enzyme inhibitors in complex mixtures for adenosine deaminase (ADA), a target involved in the adenosine inflammatory response pathway. This present study probes the limits of bioSPE using both multiple reaction monoitoring (MRM) and data dependent acquisition (DDA) mass spectrometric detection and their application to screening natural product extracts. The strong binding inhibitor EHNA (K_d = 1 nM) can be detected at concentrations as low as 10 nM using MRM and 500 nM using DDA, while the weak binding inhibitor MAC-0038732 (K_d = 3 μM) can be detected at 100 nM by MRM and 500 nM using DDA, when employing an online automated assay format. Detection limits can be reduced to 200 nM when using DDA-MS if an equilibration step is performed using offline manual loading. To examine the potential of bioSPE for natural product screening, fungal endophyte secondary metabolite extracts were then screened for binding affinity to ADA immobilized on macroporous silica columns. An offline loading method was used to isolate low affinity inhibitors, which were then detected using DDA-MS. Of the 26 extracts tested, 4 extracts showed weak non-specific binding to the column, while one extract provided two compounds that selectively bound to ADA as confirmed using IMER/MS. Compounds were isolated with m/z values of 232.2 and 292.2, which were further purified for subsequent structural characterization by NMR spectroscopy.

Introduction

Enzymes are a class of drug targets that are dysregulated in many diseases[225] and are commonly treated with small molecule inhibitors. Inhibitor screening using immobilized enzymes enables the target to be used in multiple assays, allows for wash steps, and results in enhanced signal intensity. Sol-gel derived silica surfaces are an ideal material on which to perform bio-immobilization. These high surface area materials can be cast into a wide variety of assay formats such as microarrays,[226-228] or columns,[143,206,214,229,230] and can be coupled to a wide variety of detection devices such as fluorescence,[143,226-228] or absorbance spectroscopy,[206,230] or mass spectrometry (MS).[143,206,214,229] These robust materials also provide an easy method to either physically entrap[206] or covalently couple enzymes.[229]

Enzyme-doped columns coupled with MS detection have demonstrated efficacy as a viable screening platform for the discovery and characterization of protein-ligand interactions.[8,13,16,134,143,206,229,231,232] Immobilized enzyme reactor (IMER) screening is exceptional at detecting inhibition of enzyme activity,[143,206] but is limited to enzymes with a substrate and product of different mass and are susceptible to false positives from isotope interference.[229] Bioselective solid phase extraction (bioSPE) is an orthogonal screening method that identifies inhibitors from mixtures in a quantitative manner using affinity based isolation coupled to tandem mass spectrometry. A mixture of compounds is loaded onto the column and then washed to remove non-specific binders. An extraction solvent is applied to the column to elute the inhibitor by either denaturing the protein or competing the analyte from the active site. Although sol–gel entrapment has

been used successfully for IMER studies,[128,143] it does not retain a high enough level of immobilized enzyme to perform bioSPE, as it provides insufficient analyte retention and detection.[229] Covalent immobilization of enzymes to a functionalized silica surface overcomes this problem, and provides high enzyme loading while maintaining intrinsic enzyme function. The resulting protein-doped materials have 50 – 100 fold higher protein loading, and can pre-concentrate inhibitors on the column, leading to efficient isolation of bioactives from complex matrices with a significant signal enhancement.

Using compound mixtures as a source for HTS can significantly increase sample throughput when screening bioactive chemical libraries[233] and has been demonstrated previously in our group with acetylcholinesterase[206] and more recently adenosine deaminase.[229] Our prior studies used synthetic mixtures of pure bioactives, all at the same concentration. However, preparation of such mixtures is cumbersome, and the compounds are expensive. An alternative and abundant source of structurally diverse bioactive compounds are natural product (NP) extracts, which have been widely used for generating lead compounds for drug discovery.[234] These are typically complex mixtures of lipophilic compounds that may have >100 components over a wide range of concentrations.[235] A major goal of this study was to determine if bioSPE would be amenable to screening these highly complex mixtures, though for this initial study we selected compounds that are water soluble, which are typical for orally administered drugs.[149]

Herein we apply bioSPE to a series of endophyte secondary metabolite extracts using immobilized adenosine deaminase columns to demonstrate the potential utility of

bioSPE for screening NP extracts. Hits identified in an initial bioSPE screen are subjected to a secondary IMER screen to assess inhibition of ADA function, and any validated hits are further evaluated for potency and structurally characterized by NMR spectroscopy This study provides key insights into the utility of bioSPE as a primary screening tool for NP extracts, and shows that fungal extracts may provide a useful source of compounds for modulation of ADA activity.

Materials and Methods

Chemicals. Tetramethylorthosilicate (TMOS, >99%), glycerol, 10 kDa polyethylene glycol (PEG), adenosine deaminase (3.5.4.4 from bovine spleen), 3-aminopropyltriethoxysilane (APTES), ammonium acetate (99.999%), HEPES, erythro-9-amino-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA, >98%), fluorescein, trimethoprim, adenosine (>99%) and glutaraldehyde (8% v/v in H_2O) were obtained from Sigma Aldrich. Diglycerylsilane (DGS) was prepared from glycerol and TMOS as described in detail elsewhere.[197] 250 μm i.d. fused silica capillary tubing was from Cedarlane (Burlington, ON). Bioactive compounds for screening were obtained from the High Throughput Screening facility in the Centre for Microbial Chemical Biology at McMaster University. Distilled deionized water was purified using a Milli-Q A10 Synthesis system. All other reagents were of analytical grade and used as received.

Fungal Endophyte Extraction. Fungal endophyte secondary metabolites were extracted from Canadian seed-bearing plants and are described in detail elsewhere.[236] The fungal endophyte filtrates (supernatant) had previously been extracted with ethyl acetate, dried

under nitrogen and then resuspended in ~600 µL of acetonitrile. Some of the extracts contained minimal amounts of DMSO to help resuspend the samples. 100 µL of each extract was transferred to a 2 mL HPLC vial and evaporated to dryness under nitrogen. To each evaporated sample, 250 µL of a 90:10 water-methanol solution was added. Samples were sonicated for 1 – 2 minutes followed by vortexing, repeated sonication and vortexing and then left over night at 4 ºC. The samples where then filtered using a 0.45 µm GHP syringe filter (Acrodisc), centrifuged at 10,000 × g for 3 minutes, then transferred to 2 mL HPLC vials with glass inserts. Enhanced mass spectra (EMS) were collected for each endophyte metabolite extract in order to fingerprint the metabolite peaks present. For details on sample preparation and dilution factors, see Table S4.1 in the supporting information.

Column Fabrication. Covalently bound adenosine deaminase (ADA) columns were prepared as previously described.[229] Briefly, sol-gel derived monolithic silica columns were prepared by mixing 50 µL of 225 mM HEPES, pH 7.0 with 50 µL of 20% (w/v) 10 kDa PEG and 100 µL of 1 g/mL hydrolyzed DGS. The mixture was infused into 3 m of 250 µm i.d. fused silica capillary. Columns containing covalently bound ADA were prepared as described elsewhere[29] (See SI for details), and columns were cut into 20 cm segments for bioSPE or IMER assays.

Bio-Solid-Phase Extraction Assays. All assays were performed on an AB Sciex QTrap 2000 with an electrospray ionization source using an Eksigent 1D-nanoLC and AS-1 autosampler. Channel A contained 1% acetic acid in methanol, while channel B contained 20 mM ammonium acetate, pH 7.5. Both channels operated at a flowrate of

5 µL/min. Channel A was directly connected to a tee upstream of the electrospray source while channel B was connected to the autosampler and the bioaffinity column before connecting to the tee. The mass spectrometer was operated in MRM mode for analysis of simple mass encoded mixtures or linear ion trap mode with data dependent acquisition (DDA) for mixtures of high complexity and for natural product extracts. Table S4.2 in the SI contains the MRM transitions for all monitored control compounds. BioSPE assays were performed using the same conditions for all online experiments: an 85 µL sample loop was used to load and equilibrate the mixtures on the ADA-doped column. The columns were washed with 85 µL of 200 mM ammonium acetate followed by extraction using 25 µL of 3% acetic acid. After each run, the columns were flushed with 3% acetic acid to ensure all the inhibitors were removed from the column.

For the DDA method, enhanced MS (EMS) survey scans were collected across the range of 150 → 700 m/z at a scan rate 4000 Da/s during the elution phase. A threshold value was set at 5000 cps with dynamic background subtraction enabled and all former target ions excluded. An ion exclusion list was generated from an extraction performed on a blank column and included HEPES buffer at 239.4 m/z, *n*-butyl benzenesulfonamide (plasticizer) at 214.1 and 236.1 m/z, polyethylene glycol at 217.4 m/z and a DMSO adduct at 257.1 m/z. Enhanced product ion (EPI) spectra were collected for the three most intense ions in the survey scan using collision energy of 30 V, and monitoring from 50 to 700 m/z. The resulting EPI spectra were used to help identify the compounds that were extracted during the assay. Peaks generated from MRM and DDA extracted ion

chromatograms (XICs) were Gaussian smoothed and analyzed using Analyst Software v1.5.1 and the IntelliQuan algorithm.

Evaluation of BioSPE with Complex Mixtures. The columns were assessed for their capability to isolate and identify inhibitors from mixtures of increasing complexity using MRM and DDA methods. A series of mixtures containing EHNA at concentrations of 10 nM, 100 nM, 1 μM or 10 μM were prepared, with EHNA being present in mixtures with a total of 2, 10, 100 or 1000 bioactive compounds, to determine if the potent ADA inhibitor could be isolated from each mixture. The series was repeated with the low affinity ADA inhibitor (Z)-8-chloro-6-phenylbenzo[f][1,3,5]triazocine-2,4(1H,3H)-diimine (MAC-0038732) to evaluate the effect of compound affinity on bioSPE performance.

BioSPE with Multiple Inhibitors. Mixtures with both inhibitors were also tested to see if multiple inhibitors could be simultaneously extracted from a mixture. Samples were prepared containing EHNA and MAC-0038732 at molar ratios of 1:1, 1:10 and 10:1 where 1 is 85 pmol in 85 μL (1 μM). Trimethoprim was added at a final concentration of 1 μM to all mixtures as a non-binding control, including complex mixtures with 10, 100 or 1000 compounds. The peak areas were compared to calibration curves obtained in MRM mode for both inhibitors prepared in 3% acetic acid to determine the quantity of inhibitor extracted. Mixtures were also tested using DDA to see if both inhibitors could be selected by the method during peak elution.

Mixtures with Random Concentrations & Offline Protocol. Prior to screening extracts, a mixture was prepared containing 20 different compounds at random concentrations.

EHNA was spiked into this mixture at 1 μM and 7.5 μM. BioSPE was performed on these mixtures to determine the ability to extract the inhibitor from a mixture with varying compound concentrations. An offline protocol was then used to assess whether larger volumes of a lower concentration solution would enhance analyte preconcentration, and thus provide a higher quantity of compound, aiding in detection. For this protocol, mixtures containing 100 or 1000 compounds at 200 nM each were spiked with 200 nM EHNA and prepared in 5% methanol, 2% potato dextran, 10 mM ammonium acetate, pH 7.5 and loaded onto a 10 cm ADA column segment using a syringe pump (225 μL injection volume) at an infusion rate of 20 μL/min. The columns were flushed with 60 μL of 200 mM ammonium acetate offline, followed by a 5 minute wash with the same buffer at 5 μL/min once the column was attached to the nanoLC to stabilize flow into the electrospray source. Data dependent acquisition was initiated upon injection of 3% acetic acid at 5 μL/min and continued for 12 minutes.

Endophyte Metabolite Extract Screening. A total of 26 fungal endophyte extracts were subjected to the same offline screening protocol described above. The resulting data dependent fragments that showed XIC peak areas greater than 2.0×10^8 with a Gaussian peak shape were subjected to a secondary bioSPE screen. Peaks extracted in duplicate were tested on a heat denatured ADA column to rule out non-specific binding or precipitation on the column due to low solubility. Compounds passing the screening criteria were then subjected to a functional IMER assay.

IMER Assay. For primary "hits" that showed no non-specific binding, IMER analysis[229] was used to confirm functional enzyme inhibition. A 3.25 cm segment of the ADA doped

column was used to perform the IMER assay. The nanoLC was filled with 100 μM adenosine in 20 mM ammonium acetate, pH 7.5 in channel A. A control sample containing 1 μM EHNA + 100 μM adenosine was prepared and run at the beginning and the end of the assay to confirm immobilized ADA activity. Mixtures were prepared as described in Table S4.1 and at 5 times this concentration, and then injected on column. Both samples were assessed for their product/substrate (P/S) ratios during the run to determine if inhibition was occurring (see SI for details). Extracts that showed inhibition using the IMER assay were then fractionated by HPLC, serially diluted and retested by IMER to confirm the functional components.

Results and Discussion

Evaluation of Mixture Complexity. Bioactive compound screening using mass encoded mixtures requires a significant amount of sample handling prior to assaying, but permits for significantly more compounds to be screened in a single bioSPE run. BioSPE was evaluated using mixtures with an increasing number of compounds to see if the ADA columns could isolate and identify an inhibitor from increasingly complex mixtures. A series of aqueous mixtures were prepared containing 10, 100 and 1000 known bioactive compounds and spiked with either EHNA or MAC-0038732 ranging from 10 nm to 10 μM. The minimum infusion concentration was determined for each inhibitor in both MRM and DDA modes.

In MRM mode, extracted EHNA could be detected when infused as low as 10 nM, while the lower affinity compound MAC-0038732 could be detected when infused at

100 nM. The difference in this detection limit between the two inhibitors is expected since the affinity of MAC-0038732 is much lower (K_d = 3 μM) than EHNA (K_d = 1 nM). Extraction peaks can be quantified using a calibration curve (Figure S4.1). When each inhibitor was infused individually at 1 μM, the analyte preconcentrations were 4-fold for EHNA and 2-fold for MAC-0038732, or extraction efficiencies of 72% and 36% respectively. Extraction efficiencies can give a relative measure of inhibitor affinity for the bound ADA, but it should be kept in mind that the longer the wash time and the greater the inhibitor off-rate, the lower the extraction efficiency will be. A sufficient column wash must be applied when separating inhibitors from highly complex mixtures to ensure that other analyte interactions with the stationary phase are minimized.

When using data dependent acquisition, both EHNA and MAC-0038732 could be detected at infusion concentrations of 500 nM (Figure 4.1), although MAC-0038732 was more reliably detected at concentrations ≥1 μM. Both EHNA and MAC-0038732 could be detected at lower concentrations using MRM compared to DDA. This was expected since the spectrum background noise in MRM is significantly lower than for DDA, providing better signal-to-noise levels and hence better detection limits for the MRM mode. There is an inherent amount of noise when analyzing samples of unknown composition and high mixture complexity in full range MS. Although MRM is clearly the more sensitive method, when searching for novel inhibitors in complex mixtures, MRM is not possible when the analyte of interest is unknown. Dynamic background subtraction of EMS spectra during the rise of the peak front is essential for the identification of $[M+H]^+$ ions out of the survey spectrum. This allows inhibitors to be

differentiated from contaminants that are easily ionized in the harsh wash or carried over on the stationary phase.

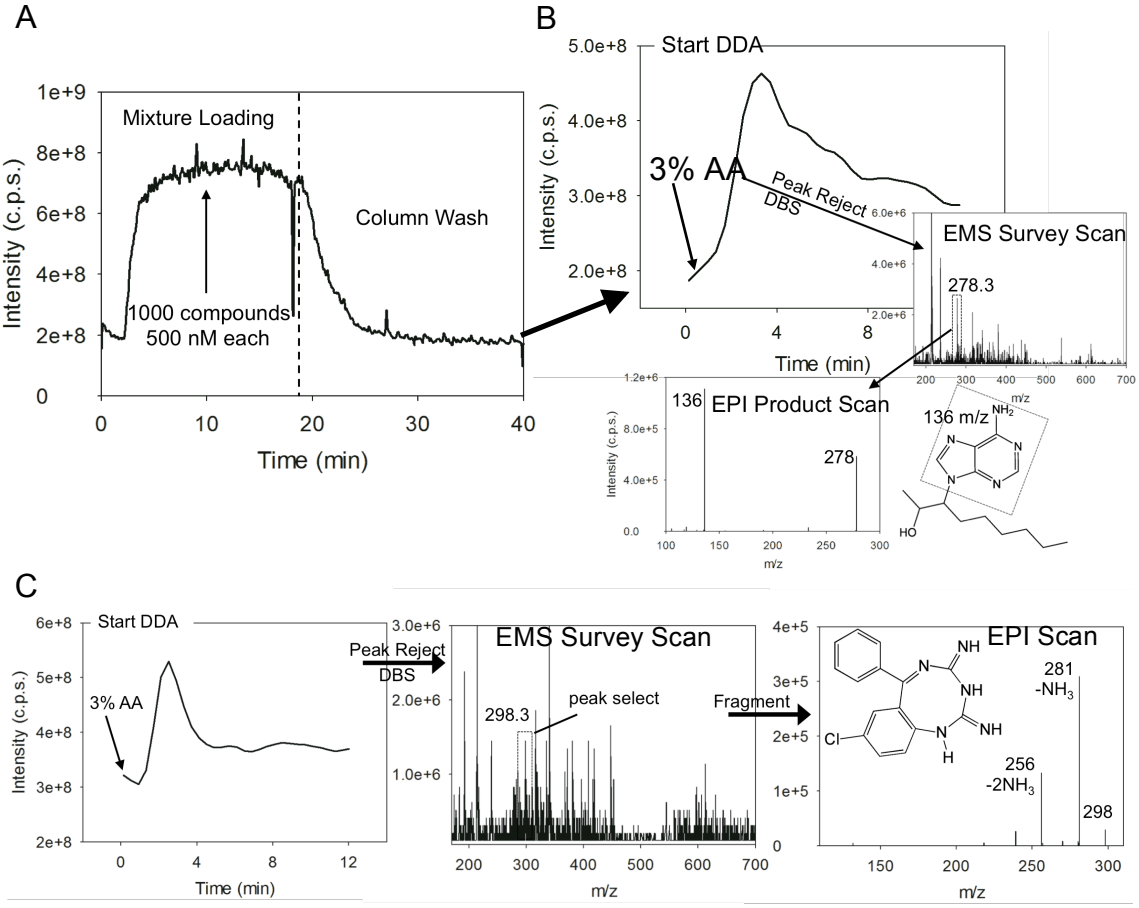

Figure 4.1. (A) Total ion chromatogram (170 to 700 m/z) for infusion of 1000 compounds on an ADA-doped column followed by a mild wash phase containing 200 mM ammonium acetate. (B) EHNA inhibitor elution using 3% acetic acid showing TIC, selected enhanced mass spectrum survey scan during peak front and enhanced product spectrum of selected peak at 278.3 m/z corresponding to EHNA. (C) MAC-0038732 elution with 3% acetic acid using the same method for EHNA resulting in EPI spectrum corresponding to expected fragmentation pattern.

BioSPE with Multiple Inhibitors. In order to determine whether more than one inhibitor could be extracted and detected simultaneously, two inhibitors with low and high affinity were infused onto the column in molar ratios of 1:10, 1:1 and 10:1 (EHNA:MAC-

0038732) with 1 being 1 μM. Both inhibitors could be detected by MRM-MS in mixtures up to 1000 compounds (1 μM per compound) in all ratios tested. Figure 4.2 shows extraction of both weak and potent inhibitors, and although both are detectable in MRM, it was clear that the more potent inhibitor, EHNA, outcompetes the weak inhibitor, MAC-0038732. For example, in the 10:1 ratio, MAC-0038732 only has an extraction efficiency of 2% while alone at a 1 μM infusion it has a 36% extraction efficiency. In DDA mode, only EHNA was selected from the survey spectrum in the 1:1 ratio and the 10:1 ratios, while both inhibitors were successfully selected in the 1:10 ratio. When manually analyzing and integrating the MAC-0038732 XICs in the 10:1 sample, small extraction peaks were still observed with an average area of $2.1 \pm 0.6 \times 10^8$. Therefore, to find a large range of inhibitor potencies in complex mixtures, a minimum area criteria of 2.0×10^8 was set as a threshold for interactions with the ADA columns when performing DDA bioSPE.

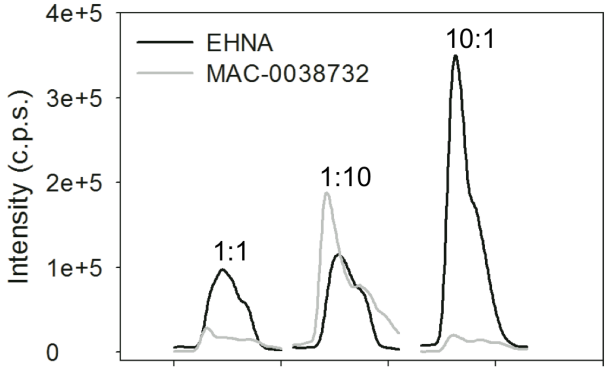

Figure 4.2. MRM chromatograms showing extraction of multiple inhibitors at EHNA:MAC-0038732 ratios of 1:1, 1:10 and 10:1, where 1 = 1 μM. Both inhibitors can be detected at all ratios tested in MRM, but EHNA outcompetes MAC-0038732 making detection of weak inhibitors in the presence of potent inhibitors more difficult.

Random Concentration Mixtures & Offline Protocol. Two randomized mixtures were tested that contained a series of compounds at various concentrations spiked with EHNA at a high (7.5 μM) and a low (1 μM) concentration. This was to test the ability of the ADA columns to extract the inhibitor in the presence of greater mixture complexity, particularly with much higher quantities of other compounds that had no interaction with the enzyme. EHNA was successfully extracted from both mixtures and could be detected using bioSPE DDA MS/MS (Figure 4.3). Compounds of higher concentration were easily washed from the column with the high ionic strength buffer and the resulting XICs collected from the DDA method were comparable in shape to the MRMs. The extraction efficiencies appeared to be low compared to previous extractions with the 1 μM EHNA infusion extracting 21 pmol from the column (24.7% efficiency), while the 7.5 μM EHNA infusion had 54 pmol extracted (8.5% efficiency). The random mixture extractions were performed on the same ADA column that had been used for the multiple inhibitor extractions and although performance was no longer optimal, identification of unknowns is still possible on complex samples after continuous and long-term use.

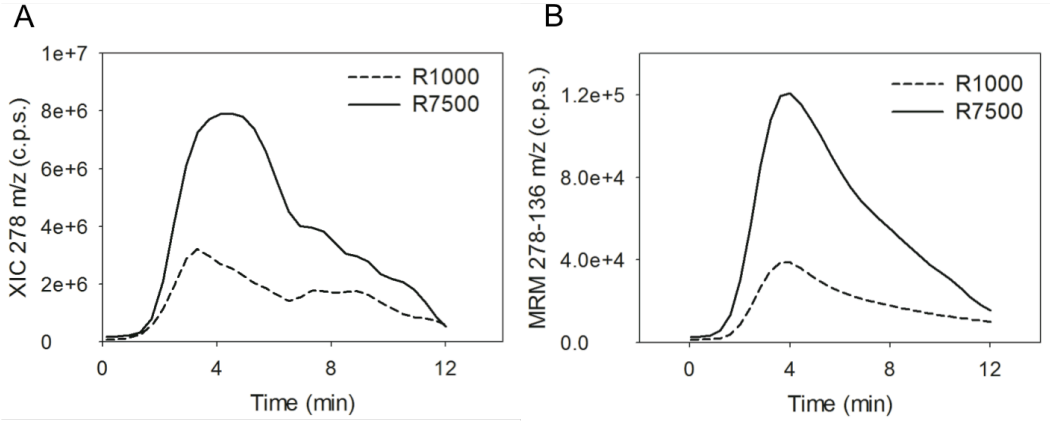

Figure 4.3. (A) EHNA elution peaks as detected by the DDA method for both the 1 μM (R1000) and the 7.5 μM (R7500) in comparison with (B) using MRM detection. Both sets of peaks are comparable in shape with the XIC peaks being larger in area and MRM peaks having better symmetry although some tailing is present.

One issue with the online assay was the relatively low infusion volume attainable with the autosampler (85 μL). Such a volume (about 17 bed volumes) may not provide saturation of the column with lower affinity compounds, and thus an offline method was used to increase infusion volume prior to washing and elution into the mass spectrometer. In the offline mode, 225 μL of the compound mixture containing either 100 or 1000 compounds at 200 nM each, including EHNA, was infused onto an ADA column using a syringe pump at a flow rate of 20 μL/min (45 bed volumes). The higher volume allows lower compound concentrations to be used to attain column saturation, and potentially a less potent wash (since the assay is targeted to lower affinity compounds). Following compound loading, the column was equilibrated online for 5 minutes using 20 mM ammonium acetate prior to the harsh elution wash in order to stabilize the total ion chromatographic (TIC) signal. Using the offline method, the DDA-MS could easily extract EHNA from both 100 and 1000 compound mixtures, resulting in extractions of 19

and 26 pmol respectively, showing that increased mixture complexity does not affect the extraction efficiency. Potential advantages of the offline assay method are larger infusion volumes, increased flow rates (shorter infusion time), the ability to use reduced mixture concentrations, the opportunity to add an incubation period prior to washing, the ability to use multiple pumps concurrently for loading, washing and extracting compounds, and the potential to extract directly into a fraction collector rather than a MS for implementation of alternative structural elucidation methods.

Determining Hit Criteria. The peaks selected by the DDA method generate an XIC and an EPI spectrum. The XICs within the elution front window must be analyzed to determine whether they show reproducible binding to the column. Figure 4.4 depicts a selection of XICs from the survey scan during EHNA extraction after offline loading in the presence of 1000 compounds prepared in a matrix containing potato dextran (the media used to grow the fungal endophytes). As shown in Figure 4.4, the peak elution front typically occurs between 2.1 and 3.8 minutes and although the DDA run time is 12 minutes long to allow a full peak to elute, minor fluctuations in the elution front can occur depending on the backpressure present on the column. The peak analysis window was set to include ions selected between 1 – 5 minutes. However, ions selected for fragmentation within this window must fit the criteria below to be deemed a hit. 1) Often during elution with 3% acetic acid, ionization efficiency increases showing an increase in signal. This results in the peak being selected by the DDA method, but no peak being resolved from the baseline. Peaks must therefore have an appropriate Gaussian profile and a minimum peak window of 60 s. as determined by the IntelliQuan algorithm in Analyst. 2) Peaks

must also be of sufficient area, greater than 2.0 x 10^8 as determined above for weak binding MAC-0038732. 3) Peaks must be reproducible in duplicate screens: Figure 4.4A illustrates this scenario during a control extraction of EHNA. The potent inhibitor is by far the most significant peak yielding an area >2.0 x 10^9 with most other ions being of disregarded based on a lack of XIC peak shape. However, two peaks are selected at 612 m/z and 183 m/z with symmetrical peak profiles and areas of 3.3 x 10^8 and 2.4 x 10^8 – both above the minimum area and peak width criteria. When the sample is repeated in Figure 4.4B, EHNA still has an area >2.0 x 10^9 but neither peaks 183 m/z or 612 m/z are present. 4) Samples that pass the previous criteria must be subjected to a negative control bioSPE assay using a heat denatured (HD) ADA column. Due to the complexity of extracts and their potential for containing contaminants such as low solubility compounds, plasticizers and DMSO, binding to functional ADA must be confirmed. Once these four criteria are met, the compound can be considered a hit and undergo further characterization.

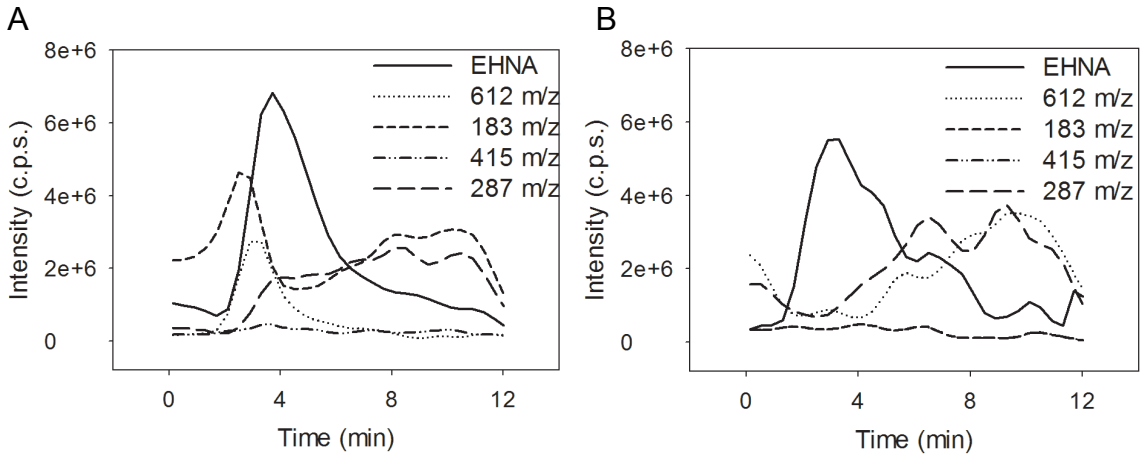

Figure 4.4. Extraction of EHNA from spiked endophyte extract matrix: (A) XICs of peaks detected by DDA method within the peak front time window. Most selected ions can be disregarded by peak shape (415 and 287 m/z) while 183 can be disregarded from early elution time and high initial signal indicating non-specific binding. Peak corresponding to 612 m/z is small but would be considered suspect (B) Replicate sample extraction shows the artifact peaks are not reproducible while the major EHNA peak elutes at the same time.

Fungal Endophyte Metabolite Screening. Since the concentration of compounds within the extracts were unknown, column loading and washing took place off-line to maximize the equilibration time of analytes with immobilized ADA and to avoid excessive contamination of the ESI source. There were a total of 26 endophyte extract samples analyzed by bioSPE-DDA-MS. Of these samples, 16 had no data dependent selected ions within the elution window based on the peak selection criteria. Ions that showed binding in the primary bioSPE screen were then subjected to a secondary bioSPE assay to ensure reproducibility. In some instances where metabolites may have precipitated or non-specifically bound to the column during equilibration, carry-over into the elution phase can occur resulting in a peak of equal or greater area on an HD-ADA column. Figure 4.5A depicts non-specific binding from a metabolite at 263.2 m/z

(Lachnochromonin A) in extract sample E-006 compared to a typical EHNA extraction peak. Non-specific elution peaks are typically selected by the DDA method within the first minute of EMS survey scans while true hits elute later with the peak elution front. Extract sample E-051 had a significant amount of interacting compounds present that required further analysis. A non-specific binding example at 276 m/z is shown in Figure 4.5B where the ion is eluted during both ADA bioSPE runs and the HD-ADA bioSPE run. Ligands that specifically interacted in duplicate with functional ADA showed no interaction with HD-ADA columns as shown in Figures 4.5C and 4.5D with ions at 232.2 and 292.2 m/z. These two compounds were therefore deemed potential hits and were selected for further screening. These data show the importance of utilizing a control column to ensure that ligands selectively bind to the active protein, and clearly demonstrate that the bioSPE method, with appropriate controls, can be used to extract novel compounds from natural product extracts.

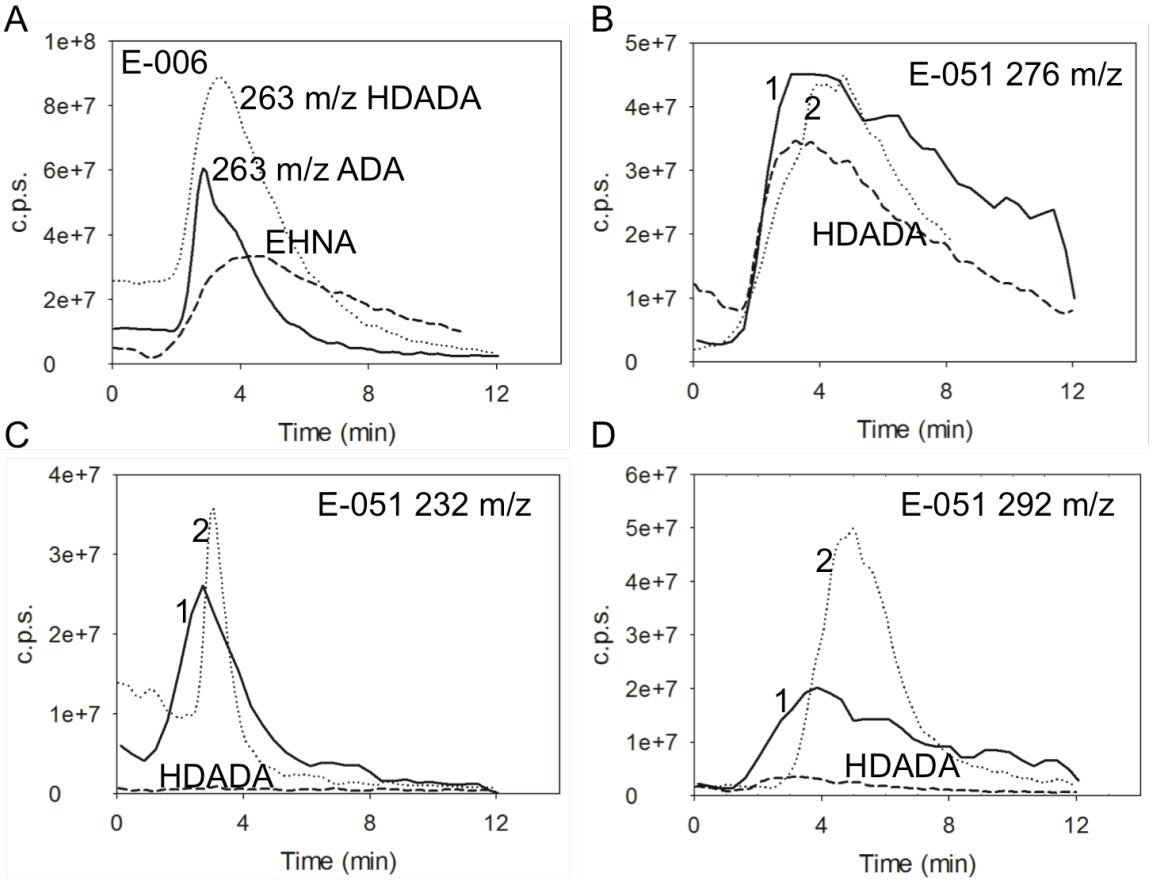

Figure 4.5. A mass peak selection criterion for determining specific binding is done by comparing extractions on ADA and HDADA columns. (A) Sample E-006 contains non-specific binding Lachnochromonin A at 263.2 m/z and is compared to a typical EHNA extraction peak. Replicate bioSPE runs from sample E-051 have selected ions compared with inactive ADA extractions for (B) non-specific binding 276.2 m/z and specific interactions from (C) 232.2 m/z and (D) 292.2 m/z.

All selected ions from the 26 extracts that passed the primary hit criteria were analyzed in duplicate and checked for non-specific binding, providing 5 extracts that showed interactions with functional ADA (Figure 4.6A) with specific extracts and ions tabulated in Table 1. Four of the extracts were clustered in the lower right quadrant of the duplicate plot, indicating that their interaction with ADA may not be as potent as EHNA

or not easily ionizable using ESI-MS. Sample E-051 contained two promising compounds that were present as minor peaks in the fingerprint spectrum (Figure 4.6B) and was therefore selected for an IMER activity assay. After the screen was complete, both a bioSPE of EHNA and an IMER with adenosine were tested on each column used to confirm the enzyme was functional throughout the screen. In all cases they showed the same, if not better, performance after the screening was complete.

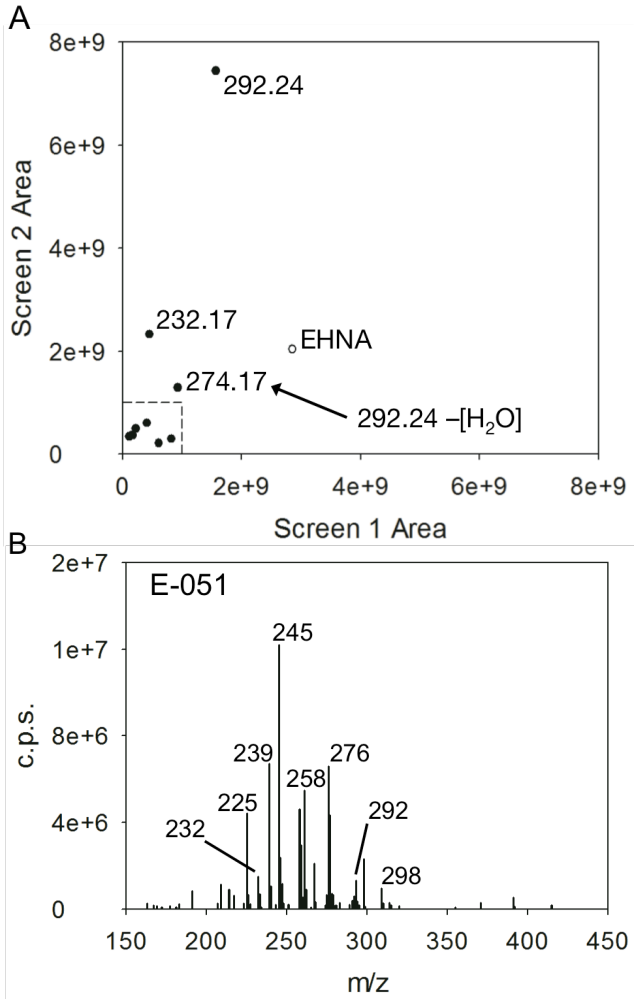

Figure 4.6. (A) Duplicate plot showing hit compounds above the screening criteria. Compounds 292.2 and 232.2 m/z were selected for further analysis since they were readily visible in (B) the enhanced mass spectrum of sample E-051. The extract was directly infused and the spectrum was solvent background subtracted. Potential hit compounds at 232.2 and 292.2 m/z are clearly seen amongst many other components.

Table 4.1. Fungal Endophyte Extract Samples and Hit Compounds From BioSPE Screen.

Extract	Hit $[M+H]^+$ (m/z)
E-006	249.21
E-051	292.24 232.19
E-111	263.14
E-202	169.14 183.01 197.06
E-222	223.26

After identifying two novel lead compounds that interacted with active ADA, it was important to determine their structures and confirm functional inhibition. Although the product ion spectra generated from the data dependent method do not unequivocally identify the compound, they give important primary structural information via the collected EPI spectra (Figure 4.7). The major fragments of 232.2 m/z are 175.2, 202.1 and 203.0 m/z, while the major fragments for 292.2 m/z are 274.2 and 256.2 m/z. The EPI spectrum for the selected peak 274.2 m/z was almost identical to that of 292.2 m/z indicating the structures were differing only by water loss. These could be two similar compounds, but more likely the 274.2 m/z undergoes labile source fragmentation during electrospray ionization. High-resolution masses have been generated for both compounds in negative mode MS. The first compound was $[M-H]^-$ at 231.1030 m/z corresponding to a molecular formula of $C_{14}H_{15}O_3$. This compound is currently being extracted from four other co-eluting compounds with preliminary heteronuclear single quantum coherence (HSQC) 2D NMR results indicating there are at least two methyl groups, one methoxy and two methylene groups. The second compound was found at an $[M-H]^-$ of

291.0876 m/z, which correspond to molecular formulas of $C_{15}H_{15}O_6$ but is low abundance in the extract with at least one structural isomer and was not available in a high enough quantity to perform 2D NMR. More of these compounds are currently being grown and extracted for further study. BioSPE was capable of detecting these ADA inhibiting compounds from the fungal endophyte extracts in the presence of greater quantities of non-inhibiting compounds, making it an excellent platform for detecting protein-ligand interactions and capable of generating lead compounds for further study.

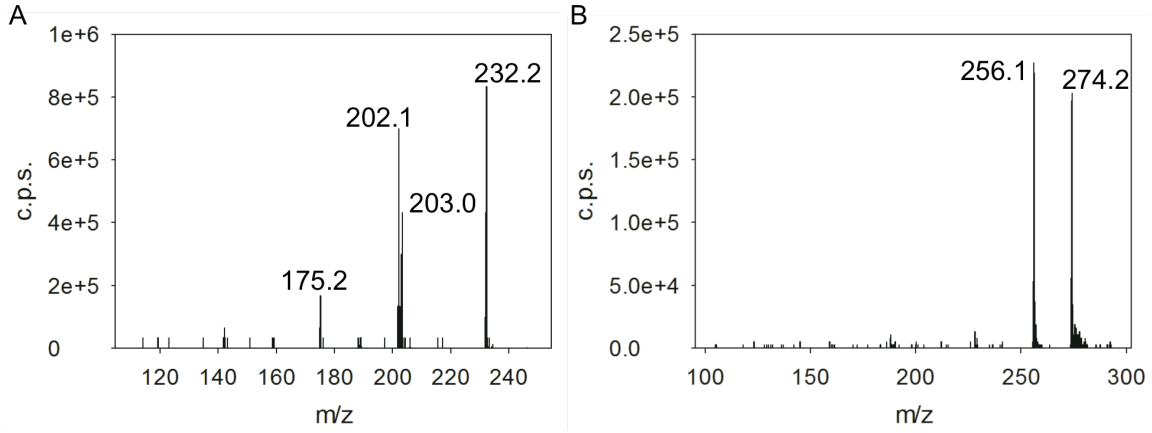

Figure 4.7. EPI spectra of ions showing interaction with functional ADA (A) 232.2 m/z and (B) 292.2 m/z. Both lead compounds are novel and uncharacterized. EPI fragmentation patterns provide preliminary structural information.

IMER Assay. To ensure that the extracts contained functional inhibitors of ADA, an IMER assay was performed on sample E-051 at two different concentrations. Although absolute molar concentrations were unknown, they were estimated from the fingerprint spectrum based on comparison to the signal intensity of a sample of 100 μM adenosine (assuming that these compounds are adenosine analogs with similar ionization efficiencies to adenosine). Based on this assumption, ions 232.2 m/z and 292.2 m/z were

estimated to be present at approximately 0.6 μM and 0.2 μM in the low concentration dilution and 3 μM and 1 μM in the high concentration dilution. Upon screening, both concentrations showed ADA inhibition, producing 60% activity in the low concentration sample and 23% activity in the high concentration sample (Figure 4.8A). This assay confirms that an ADA inhibitor is present in sample E-051.

To further confirm compound identity and function, HPLC purification of E-051 was performed to isolate the compounds found at 232.2 m/z and 292.2 m/z for functional inhibition. A total of 7 fractions were produced, each of which were serially diluted into a concentration gradient ranging from 25 to 630 μg/mL. The IMER assay was used to assess functional inhibition on each concentration gradient. Fraction 4 and 6 had the greatest inhibitory affect (Figure 4.8B) with activities reaching 82% for fraction 4, which contained the low abundance compound at 292.2 m/z, and 18% for fraction 6 containing the compound at 232.2 m/z; fraction 5 contained overlap of both the 292.2 and 232.2 m/z compounds and also had a similar inhibitory affect as fraction 4 ~80% (not shown). Fraction 1 is also depicted in Figure 4.8B with an increase in activity owing to greater ionization of the product in the presence of methanol (12.5% at highest concentration). All other fractions showed similar activity profiles to fraction 1. Fraction 4 contained at least two isomers of the 292.2 m/z compound and was present with at least one other co-eluting compound, which may explain why inhibition was not as great as anticipated. The 232.2 m/z compound present in fraction 6 was of higher abundance and capable of greater inhibition but present with at least 4 other co-eluting compounds. Work is

ongoing to further purify these compounds in larger quantities to determine the inhibition constant (K_I) and for structure elucidation by 2D NMR.

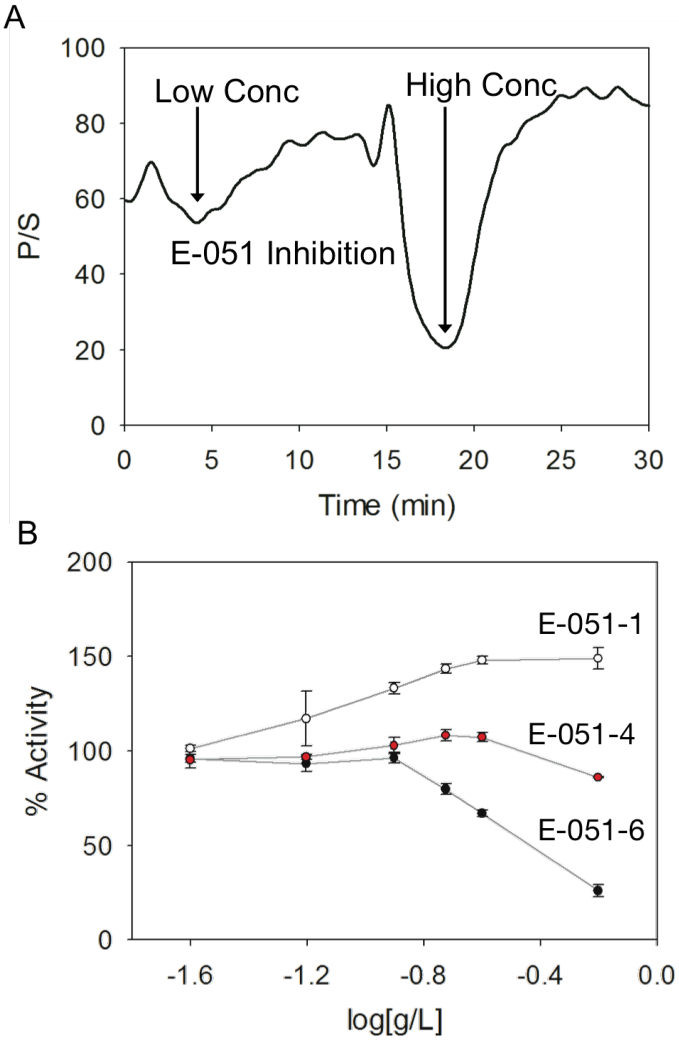

Figure 4.8. Immobilized enzyme reactor screen of (A) E-051 showing concentration dependent functional inhibition of ADA. This confirms that a functional inhibitor is present in this extract sample. (B) % Activity levels assessed by IMER on E-051 fractions 1, 4 and 6 demonstrating functional inhibition of fraction 4 to 82% activity containing low abundance compound at 292.2 m/z and fraction 6 to 18% activity containing compound at 232.2 m/z.

Conclusion

This study demonstrates that bioSPE is capable of rapid isolation and identification of protein binding ligands present in highly complex mixtures and natural product extracts. The major benefit to this method is the ability to screen an unpurified extract in one assay, with the ability to extract both high and low affinity inhibitors. Extracted ligands could be confirmed for specific binding using heat denatured protein columns, and it should be possible to determine structures of hits by comparison with expected fragmentation patterns from HPLC-MS/MS,[237] or by NMR spectroscopy[238] database libraries. Novel compounds will likely require further isolation from the extract, which can be achieved using HPLC-MS as the high resolution compound m/z is known, and verification of the interaction can be performed with a more purified form of the metabolite. The bioaffinity column itself could also be scaled up and used for offline extraction and compound collection, which should provide sufficient compound for NMR spectroscopy to unequivocally identify the structure. For future studies, it may be possible to add a second-dimensional LC after the bioSPE step to further purify extracted compounds and aid in structural identification. Overall, the bioSPE method provides a valuable method to isolate bioactive compounds from mixtures, and should be a useful addition to the arsenal of methods for identifying important lead compounds in natural product extracts.

CHAPTER 4. SUPPLEMENTARY INFORMATION

Experimental Information

Covalent Coupling of ADA to Columns. Glycerol and PEG were removed from the column by flushing 50% methanol/water via a Harvard syringe pump. The silica surface was then flushed with neat ethanol and then infused with a 5% APTES solution in ethanol and reacted for 2 hours. Residual APTES was removed with 10 mM HEPES pH 7.5 followed by infusion with a 4% glutaraldehyde solution in the same buffer and reacted for 2 hours. Residual glutaraldehyde was removed, followed by infusion of 84 μM ADA in buffer. The ADA was allowed to react overnight at 4ºC and then removed by flushing with buffer. Remaining aldehyde functionality was capped by reaction with 500 μM glycine for 2 hours. A final buffer flush was performed prior to coupling to the LC to flush with 20 mM ammonium acetate, pH 7.5 before connecting to the MS.

Preparation of Endophyte Extracts. The 26 endophytic fungi extracts were prepared as families of compounds from individual endophytes. The metabolites present in each extract were of unknown concentration, and most were uncharacterized. A fingerprint mass spectrum of each extract was collected using a 1:50 dilution in 20 mM ammonium acetate and a make-up flow of 1% acetic acid in methanol. The base peak of each extract was compared to 100 μM adenosine prepared in the same matrix. Further dilutions were made to obtain approximately 5 μM of the base peak. This allows for sufficient dilution of the major component without excessive dilution of minor components. Dilution factors are tabulated in Table S4.1.

Table S4.1. Endophyte Extract Dilutions

Sample	Base Peak (m/z)	Base Peak Max Signal (c.p.s.)	Approx. Conc. (μM)	Dilution Factor
adenosine	268	6.5E+06	100	20
E-006	263	3.1E+07	477	83
E-024	225	6.1E+06	94	19
E-035	205	4.9E+06	75	15
E-046	245	2.9E+06	45	9
E-051	245	3.9E+06	60	12
E-052	293	2.9E+06	45	9
E-053	313	4.6E+06	71	14
E-075	189	8.8E+07	1354	250
E-083	217	2.2E+06	34	7
E-101	195	1.4E+07	215	42
E-111	203	2.4E+06	37	7
E-122	198	1.1E+07	169	36
E-138	211	4.5E+06	69	14
E-154	169	2.1E+07	323	63
E-169	293	3.4E+06	52	10
E-182	235	1.1E+07	169	36
E-184	165	1.9E+07	292	63
E-185	387	1.5E+06	23	5
E-189	241	1.4E+07	215	42
E-196	211	2.0E+06	31	6
E-200	235	6.8E+06	105	21
E-202	155	1.8E+07	277	50
E-222	313	5.1E+06	78	16
E-224	241	1.4E+07	215	42
E-257	275	3.8E+06	58	12
E-261	155	6.7E+06	103	21

Table S4.2. MRM Transitions for Control Compounds

Compound	Precursor (m/z)	Fragment (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
Adenosine	268.3	136.1	45	10	26	3
Inosine	269.1	137.1	45	10	26	3
EHNA	278.3	136.1	68	10	30	3
MAC-0038732	298.3	281.1	85	10	26	4
Trimethoprim	291.2	230.0	80	10	33	3

Calibration Curves. Both EHNA and MAC-0038732 were prepared in a matrix mimicking the elution conditions of bioSPE: 10 mM ammonium acetate with 1.5% acetic acid. A 15 µL sample loop was used to inject compounds directly into the MS and these were analyzed in MRM mode. The resulting peak areas were analyzed in Analyst v1.5.1 and calibration curves were prepared in Microsoft® Excel (Figure S4.1). Internal standards could not be used due to the extraction protocol not being able to retain non-inhibitors.

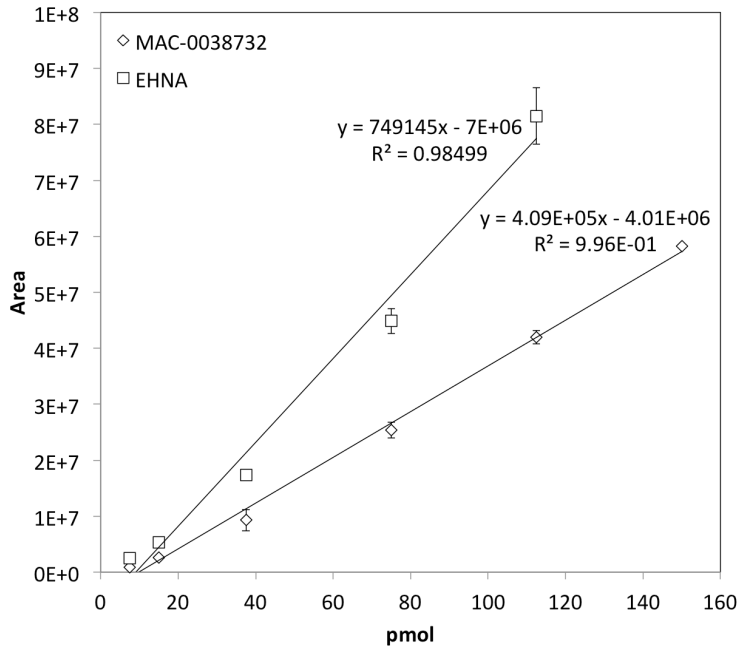

Figure S4.1. Calibration curves for EHNA and MAC-0038732. Quality control sample of 50 pmol is run once per week.

IMER Analysis. The data analysis for the IMER assay was performed as previously described.[17] Columns were equilibrated with the mobile phase to obtain an initial P/S signal ratio based on turnover of adenosine in the mobile phase to inosine by immobilized ADA. The activity of each mixture was measured by determining the P/S signal ratio in the presence of the mixture and then normalizing this to the initial P/S signal ratio prior to injection of the mixture, as shown in equation S4.1:

$$\%Activity = \frac{(P/S)_{sample}}{(P/S)_{initial}} \times 100 \qquad \text{S4.1}$$

Results and Discussion

HPLC of Endophyte Extracts. Initial assessment of endophyte extract mixture complexity was also performed by HPLC separation on a C18 column coupled to a Bruker Maxis 4G. E-051 showed the most potential interactions with ADA and the RPLC trace is shown below in Figure S4.3. The major bioactive metabolite present is Ascochitine at +277 m/z and 18.7 min, which had no interaction with either ADA or HD-ADA columns. Both compounds detected in the bioSPE assay in positive mode were analyzed under negative mode with this HPLC system and were detected at -231 m/z at 12.6 min and -291 m/z at 12.9 min. The low abundance -291 m/z peak showed the greatest binding interaction with the ADA column and both interacting compounds detected are suspected to be novel but require further structural characterization.

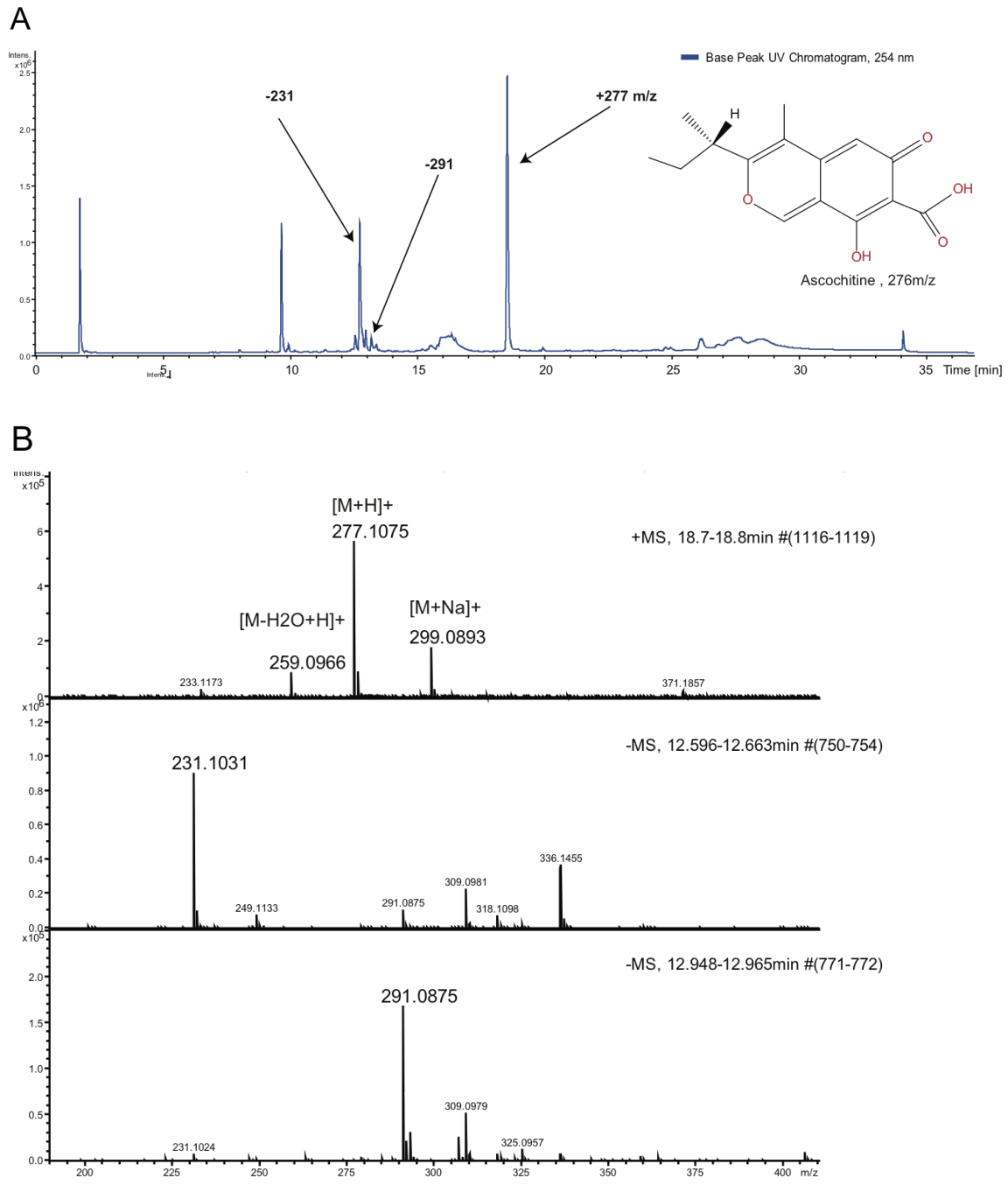

Figure S4.2 (A) HPLC chromatogram and (B) selected mass spectra of E-051 showing the major bioactive metabolite ascochitine present at +277 m/z and 18.7 min that did not bind to active ADA, -231 m/z at 12.6 min and -291 m/z at 12.9 min, both of which are uncharacterized novel metabolites that showed binding to ADA in the bioSPE assay.

CHAPTER 5. CONCLUSION

The use of sol-gel derived monolithic columns with immobilized enzymes allows for both functional and affinity-based screening of inhibitors using nanoflow LC/MS/MS instrumentation. These methods are particularly useful for screening of mixtures, and have significant potential for identification of bioactive compounds in synthetic mixtures and natural product extracts.

In the first chapter of this research, immobilized enzyme reactor columns containing AChE were optimized for screening synthetic mixtures for the presence of inhibitors. Columns were moved from a coupled reaction with the product that provided colourimetric detection to a new method using analyte-specific mass spectrometric detection. MRM mode was especially useful in preventing potential interferences from compounds being screened and also added an intrinsic internal standard by monitoring the P/S ratio. A statistical Z'-assay was performed using the known inhibitor galanthamine to assess the screening window, which indicated the assay was able to efficiently differentiate hit compounds from non-binders. A screen of 1040 compounds was performed using 52 mixtures in duplicate. This decreased the time it took to perform the screen by 20-fold. Two hit mixtures were identified during the IMER screen and manual deconvolution of mixtures was performed to identify the compounds responsible for inhibition. These were known inhibitors physostigmine and 9-aminoacridine. This study also illustrated the ability to quantify inhibitor potency by an automated IC_{50} analysis on-column that was in good agreement with solution values.

Although the IMER screening method was excellent at detecting inhibitors in synthetic mixtures, it was not capable of unequivocally identifying the inhibitor in the mixture. Manual deconvolution of mixtures was slow and required a great deal of sample preparation. To address this, a new bioSPE method was developed to rapidly deconvolute mixtures in one assay, providing an orthogonal method to confirm hits from IMER screening. In this work, the model enzyme system was changed to ADA in order to demonstrate the applicability of IMER to another enzymatic system. The use of MS/MS to detect substrate turnover was even more important with this enzyme as there is no reliable colourimetric detection method that can be used for inhibitor screening. An IMER screen was performed with the same collection of bioactive mixtures as in the previous study in order to create a suitable comparison. Three hit mixtures were identified in the screen, this time mixtures were deconvoluted both manually by IMER and in a single assay by bioSPE. One mixture contained a weak binding inhibitor, MAC-0038732, which was identified clearly by biasing the screen towards low affinity ligands. The other two mixtures proved to contain false positives as they had mass spectral interferences that interfered with detecting substrate turnover. This was confirmed first by IMER IC_{50} analysis, showing the complementary nature of the two assay formats. BioSPE was also employed to determine the presence of hit compounds in one simple experiment, which successfully identified both the control compound, EHNA, and MAC-0038732, while no interaction was observed with the mixtures containing false positives. BioSPE was also used in conjunction with a data dependent acquisition MS

method to effectively isolate and identify the inhibitor without the need for mass encoded mixtures.

A final study was performed to apply bioSPE to highly complex mixtures, including a series of secondary metabolite extracts for screening against ADA. The bioSPE assay was first probed with a series of mixtures to determine limits of detection and mixture complexity using both the low affinity ligand MAC-0038732 and the high affinity ligand EHNA. When the inhibitor has a known MRM transition, it can be detected in a more sensitive manner than by data dependent acquisition. EHNA could be detected as low as 10 nM while MAC-0038732 was detected at 100 nM using MRM-MS in mixtures containing up to 1000 bioactive compounds. Using data dependent acquisition, both of these compounds had to be selected out of a significant amount of baseline noise, increasing the limit of detection for both inhibitors to 500 nM. Moving the column loading and washing offline and increasing the infusion volume, and decreased this detection limit to 200 nM. Offline sample loading also allowed for multiple pumps to be used simultaneously, decreasing the analysis run time and increasing sample throughput while preventing contamination of the MS system, thus allowing for far more complicated mixtures to be assayed. In order to screen secondary metabolites from partially purified fungal endophyte extracts, a set of criteria was established to ensure elution peaks were specific to the functional enzyme. This included peak shape, width and retention time window. Samples that showed peaks above these criteria were ran in duplicate and on a heat denatured ADA column to verify the interaction.

The screen resulted in the discovery of two compounds, with $[M+H]^+$ peaks of 232.2 m/z and 292.2 m/z, that showed specific binding to ADA. As further confirmation of inhibition, an IMER screen was performed on the hit extract sample at a low and high dilution, resulting in reduced activities of 23% and 60% respectively. Extract E-051 was further fractionated and analyzed by IMER resulting in fraction 4 containing 292.2 m/z giving 82% activity and fraction 6 containing 232.2 m/z giving 18% activity. High resolution MS provided molecular formulas of $C_{15}H_{15}O_6$ for 292.2 m/z and $C_{14}H_{15}O_3$ for 232.2 m/z with more structure elucidation and inhibitory characterization ongoing. This work illustrated the ability of bioaffinity columns to be applied to both bioSPE and IMER to successfully screen natural product extracts for the discovery of novel protein-ligand interactions.

BioSPE technology is a direct method for detecting protein-ligand interactions, which may be applicable to other clinically relevant proteins. It is rapid and can isolate inhibitors from complex secondary metabolite extracts and provide preliminary structural information for novel compounds. Function-based screening using IMER is also an important orthogonal method for inhibitor screening, as hits can be confirmed in a complimentary method. IMER does have the drawback of not being amenable to non-enzymes, such as receptors – proteins with signaling binding sites that do not alter the ligand. BioSPE, however, has the potential to be applicable to any protein that binds a target bioactive compound.

Moving to covalent attachment was originally done to compensate for protein leaching from the monolithic silica material. This type of immobilization also allows for

screening of protein-protein interactions since the target protein is immobilized on the surface of the silica and not entrapped within enclosed pores that are not accessible. Although this is a potential loss of untapped surface area, the covalent linkage prevents significant leaching indicating that much of the surface is accessible owing to the macroporous structure. However, for application to more labile proteins, covalent bonding leaves the target susceptible to proteases or easy denaturation of tertiary structure affecting active site functionality, again illustrating the need for development better immobilization strategies.

Another important issue is that many clinically relevant targets require buffer conditions and cofactors that are not amenable to the low ionic strength eluent required for electrospray ionization methods. This becomes imperative when studying targets that require metal cation cofactors. This issue can be addressed either by using MALDI-MS, where higher concentrations of these compounds are tolerated, or 2D-LC-ESI-MS, where a reverse phase column can be used after the bioaffinity column to perform on–line desalting or separation of analytes prior to MS analysis. Such methods may ultimately allow for a much wider range of species to be used for affinity based screening and will undoubtedly provide new opportunities for HTS and drug discovery.

Immobilized enzyme columns have utility in both function and affinity based screening methods. They can be used to screen for hit compounds to generate lead drug compounds, characterize binding and enzymatic turnover kinetics and are capable of screening highly complex mixtures including natural product extracts. This technology has great potential to be further developed, particularly when coupled with analytical

instrumentation like tandem mass spectrometry and nuclear magnetic resonance spectroscopy.

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