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Frontal Affinity Chromatography with Mass Spectrometry Detection for Probing Molecular Interactions

A New Mechanistic Approach for the Development of Detection & Identification and Medical Countermeasures Against CB Agents

Nora W.C. Chan
Defence R&D Canada – Suffield

Technical Memorandum
DRDC Suffield TM 2005-173
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Abstract

Frontal affinity chromatography with mass spectrometry detection (FAC-MS) is a powerful technique for the study of molecular interactions between receptors (proteins, antibodies, enzymes) and ligands (small molecules, antigens, drug compounds). Such interactions are fundamental to biochemical pathways, from the detection of a protein cascade involved in a disease state, to the study of the disease mechanisms, to high-throughput screening for the identification and optimization of lead compounds for drug discovery. FAC-MS is able to locate and characterize key molecular recognitions in complex biological systems that are otherwise "hidden" from current technologies. Thus, this cutting-edge technique can steer biotechnological research through key areas and eliminate many years of "hit-and-miss" experimentation. It will allow for rational design and development in areas, such as bio-detection and identification, and medical countermeasure, which are currently of interest to the CF and DRDC. FAC-MS is amenable to high-throughput screening of large collections of compounds simultaneously. Currently, no other alternative method can provide unbiased, direct measures of binding affinity in a rapid and easy to perform manner as FAC-MS. This article outlines the plan of adapting FAC-MS from industry (Protana Inc.) to DRDC Suffield for defence research incorporating toxin research, drug discovery, recombinant antigen-antibody optimization and proteomics.

Résumé

La chromatographie par affinité frontale effectuant une détection avec la spectrométrie de masse (CAF-SM) est une technique puissante utilisée pour l'étude des interactions moléculaires entre récepteurs (protéines, anticorps, enzymes) et ligands (petites molécules, antigènes, composés de médicaments). De telles interactions sont fondamentales pour comprendre les voies biochimiques, qu'il s'agisse de la détection d'une cascade de protéines ayant causé l'état de maladie, de l'étude des mécanismes des maladies ou encore de la sélection à haut rendement pour l'identification et l'optimisation des composants les plus prometteurs pour la découverte des médicaments. La technique CAF-SM est capable de localiser et de caractériser les reconnaissances moléculaires clés dans des systèmes biologiques complexes qui seraient autrement « cachées » des technologies actuelles. Cette technique d'avant-garde peut ainsi aiguiller la recherche biotechnologique à travers les domaines clés et éliminer beaucoup d'années d'expérimentation « à l'aveuglette ». Ceci permettra de rationaliser le concept et la mise au point dans des domaines tels que la bio-détection et l'identification ainsi que celui des contre-mesures médicales qui intéressent actuellement les FC et RDDC. La technique CAF-SM est susceptible de faire une sélection à haut rendement de collections importantes de composés simultanément. Aucune autre méthode ne peut actuellement procurer des mesures directes et impartiales d'affinités de liaison d'une manière aussi rapide et aussi facile à effectuer que la méthode CAF-SM. Cet article souligne le plan visant à adapter la méthode CAF-SM de l'industrie (Protana Inc.) à RDDC Suffield pour la recherche en défense qui incorporerait la recherche sur les toxines, la découverte de médicaments, l'optimisation antigène-anticorps recombinant et la protéomique.

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Executive summary

Introduction: Molecular interactions between two or more biomolecules are fundamental to a wide range of research areas from the development of a biosensor, to all stages of drug discovery, including study of disease mechanisms to identify protein targets, high throughput screening for the identification and optimization of lead compounds. Many technologies exist to characterize molecular interactions, such as radioassay, microcalorimetry, enzyme-linked immunosorbent assay (ELISA), and surface plasmon resonance (SPR). Currently, no single approach has proven sufficient to support the full range of inquiry into interaction events. Frontal affinity chromatography with mass spectrometry (FAC-MS) provides a fast, versatile investigation of a range of important molecular interactions, as well as a proteomic approach to understanding how chemical, biological (CB) agents exert their toxic effects. FAC-MS can provide rapid, unbiased knowledge in binding interaction between receptor and its ligand(s) without the requirements of labelling. It can measure binding strength at true equilibrium conditions. FAC-MS has an intrinsic ability to separate bound versus unbound molecules with the addition of molecular weight identification, thus it is useful in the drug discovery and optimization process, and in identification of new protein targets against CB agents for both new strategies in biological detection/identification and medical countermeasure. This capability provides a good foundation for rational designs in new drugs and detection/identification methods against CB agents. This article outlines the plan under the Technology Investment Fund (TIF) of adapting FAC-MS for defence research incorporating toxin research, drug discovery, recombinant antigen-antibody optimization and proteomics.

Frontal affinity chromatography is a separation technique that is used to study receptor-ligand binding interaction at equilibrium. The chromatographic separation is based on the strength of the receptor-ligand interaction. In addition, detection with mass spectrometry provides molecular weight information, making FAC-MS amenable to multi-ligand binding analyses. Competition between all interacting compounds is evident by the transient overconcentration feature in the analysis, due to a displacement of a bound ligand by a stronger ligand toward the immobilized protein. Dissociation constant (K_d) between each receptor-ligand pair can be determined in a single experiment. Simple fluidic system involves syringe pumps and a fluid switching valve before an affinity column, and eluent will be mixed with organic solvent prior to spraying into a mass spectrometer.

Significance: This FAC-MS capability provides a good foundation for rational designs in new drugs and detection/identification methods against CB agents. The proteomic approach to determine unknown cell surface receptor to CB agents using FAC-MS is an essential part of a project arrangement in proteomics method development among Canada, the Netherlands, and Sweden under the CA/NL/SE Cooperative Science and Technology Memorandum of Understanding (MOU).

Vaccine development is an important area of prophylactic treatment to prevent injuries due to CB agents. Many vaccines are not specific or as effective as one would hope. To develop an effective and specific vaccine against CB agents, detailed information from mapping antigenic epitopes is necessary. Systematic mapping requires many iterations of ELISA and

western blot experiments to identify fragments that were antigenic epitopes. FAC-MS can streamline this process by reducing the requirement for expensive reagents and time-consuming experimentations.

Research towards finding medical countermeasures against botulinum neurotoxin (BoNT) is important due to the toxin's highly toxic nature. The vaccine against BoNT is losing its effectiveness over time. Besides the inadequate effects, vaccines have suffered poor safety profiles and usually require multiple injections to achieve protection. Thus, a new strategy for the development of effective countermeasures to botulism poisoning is necessary. Recent efforts focussed on inhibitors against the endopeptidase domain of botulinum neurotoxin. FAC-MS can be used to screen massive numbers of peptide mimetics against the toxin for potential drug candidates. Moreover, FAC-MS can be used to identify the cell surface receptor that recognizes the toxin. The receptor can be used as target for drug discovery to prevent entry of the toxin into the cell, but also act as recognition site for new biological detection and identification technology. FAC-MS can also be used to determine the protein cascade involves in L-thiocitrulline (L-TC) protection against sulphur mustard (HD) for better understanding of the pathway involved in toxicity. This can lead to further development of medical countermeasures against HD since there is no antidote, nor a clear understanding of its biochemical mechanism of action.

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Sommaire

Introduction : Les interactions entre deux ou plusieurs bio-molécules sont fondamentales à un large éventail de domaines de recherches allant de la mise au point d'un bio-détecteur à toutes les étapes de la découverte des médicaments dont l'étude des mécanismes des maladies pour identifier les protéines cibles et la sélection à haut rendement pour l'identification et l'optimisation de composants les plus prometteurs. Beaucoup de technologies existent pour caractériser les interactions moléculaires telles que le dosage radiologique, la microcalorimétrie, les dosages immunoenzymatiques (ELISA) et la détection par résonance plasmodique de surface. Il n'existe actuellement pas de méthode unique capable de soutenir suffisamment la gamme complète d'enquêtes des évènements d'interaction. La chromatographie d'affinité frontale au moyen de la spectrométrie de masse (CAF-SM) procure une méthode d'enquête rapide et polyvalente de toute une gamme d'interactions moléculaires importantes ainsi qu'une méthode protéomique aidant à mieux comprendre comment les agents chimiques et biologiques (CB) exercent leurs effets toxiques. La technique CAF-SM peut procurer rapidement une connaissance impartiale de l'interaction de liaison entre le récepteur et son ou ses ligands sans nécessiter de marquage. Elle peut mesurer la force de liaison dans des conditions d'équilibre véritable. La CAF-SM possède une capacité intrinsèque à séparer les molécules liées des molécules non-liées avec l'addition de l'identification du poids moléculaire ; elle est donc utile à la découverte des médicaments et au processus d'optimisation ainsi qu'à celui de l'identification des nouvelles protéines cibles contre les agents CB pour les deux nouvelles stratégies de la détection / identification biologique et de la contre-mesure médicale. Cette capacité procure une bonne fondation aux concepts rationaux des nouveaux médicaments et des méthodes de détection /identification contre les agents CB. Cet article souligne le plan du Fonds d'investissement technologique (FIT) d'adapter la CAF-SM à la recherche en défense en incorporant la recherche sur les toxines, la découverte des médicaments, l'optimisation antigène-anticorp recombinant et la protéomique.

La chromatographie d'affinité frontale est une technique de séparation qui est utilisée pour étudier l'interaction de liaison entre le récepteur et ligand en position d'équilibre. La séparation chromatographique est basée sur la force de l'interaction récepteur-ligand. De plus, la détection au moyen de la spectrométrie de masse procure des renseignements au sujet du poids moléculaire, ce qui rend la méthode CAF-SM utile aux analyses de liaisons de multiples ligands. La compétition entre tous les composés qui interagissent est rendue évidente par le caractère transitoire de la surreprésentation dans l'analyse, ceci à cause du déplacement du ligand lié par un ligand plus fort vers une protéine immobilisée. La constante de dissociation (K_d) entre chaque paire récepteur-ligand peut être déterminée durant une seule expérience. Un système fluidique simple comprend des pompes et une valve de commutation de fluides avant une colonne d'affinité et un éluant sera mélangé avec un solvant organique avant la pulvérisation dans un spectromètre de masse.

La portée des résultats : La capacité de cette technique CAF-SM est une bonne fondation pour les concepts rationnels des nouveaux médicaments et des méthodes de détection /identification d'agents CB. La méthode protéomique visant à déterminer le récepteur inconnu de surface cellulaire des agents CB qui utilisent la technique CAF-SM est une partie

essentielle d'un système de classement dans la mise au point de la méthode protéomique au Canada, Pays-Bas et en Suède selon le Protocole d'entente (PE) pour la coopération en science et technologie entre le CA/NL/SE.

La mise au point de vaccins est un domaine important du traitement prophylactique qui prévient les blessures causées par les agents CB. Beaucoup de vaccins ne sont pas aussi spécifiques ni aussi efficaces qu'on avait espéré. Pour mettre au point un vaccin efficace et spécifique contre les agents CB, il est nécessaire d'obtenir des renseignements détaillés à partir des représentations cartographiques des épitopes antigéniques. La représentation cartographique systématique requiert beaucoup de répétitions d'ELISA et de transferts Western pour identifier les fragments qui étaient des épitopes antigéniques. La technique CAF-SM peut simplifier ce processus en réduisant les besoins en réagents coûteux et en expérimentations aux durées prolongées.

La recherche axée sur la découverte de contre-mesures médicales contre la neurotoxine botulique (BoNT) est importante à cause de la nature très toxique de la toxine. Le vaccin contre BoNT perd de son efficacité avec le temps. En plus des effets inadéquats, les vaccins souffrent de profils peu sécuritaires et requièrent normalement de multiples injections pour assurer une protection. Il est donc nécessaire de découvrir une nouvelle stratégie permettant de mettre au point des contre-mesures efficaces contre l'empoisonnement par botulisme. Des efforts récents ont été axés sur des inhibiteurs contre le domaine endopeptidase de la neurotoxine botulique. La technique CAF-SM peut être utilisée pour sélectionner des nombres massifs des petptides mimétiques contre la toxine pour découvrir des candidats potentiels de médicaments. De plus, la technique CAF-SM peut être utilisée pour identifier les récepteurs de surface cellulaire qui reconnaissent la toxine. Le récepteur peut être utilisé comme cible pour la découverte de médicament pour empêcher l'entrée de la toxine dans la cellule mais peut aussi agir comme site de reconnaissance pour la nouvelle technologie de détection et d'identification. La technique CAF-SM peut aussi être utilisée pour déterminer la cascade de protéines comprise dans la protection en L-thiocitrulline (L-TC) contre l'ypérite pour mieux comprendre la voie conduisant à la toxicité. Ceci peut amener à approfondir la mise au point des contre-mesures médicales contre l'ypérite puisqu'il n'existe ni antidote ni une vision claire de ses mécanismes biochimiques d'action.

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Introduction

The understanding of molecular interactions is fundamental to the development of a biosensor that detects binding events between two or more biomolecules; and of all stages of drug discovery, including study of disease mechanisms, high-throughput screening for the identification of lead compounds, and optimization of lead compounds. The goal of most binding assays is to generate a signal that is representative of a binding event between biomolecules, i.e. ligand and receptor. This approach has become one of the most important and versatile methods for understanding the function of proteins in both biosensor and drug discovery areas.

Numerous mature and sensitive technologies for thermodynamic and kinetic characterization of molecular interactions exist, such as radioassay, enzyme-linked immunosorbent assay (ELISA), microcalorimetry, and surface plasmon resonance (SPR). No single approach has proven sufficient to support the full range of inquiry into interaction events. Newer techniques such as microarray or protein chips are used to probe molecular interactions of DNA and protein in a multiplexed format. This article presents a new concept in affinity chromatography with mass spectrometry detection for the study of molecular interactions.

Frontal affinity chromatography with mass spectrometry detection (FAC-MS) is a powerful technique for the study of molecular interactions between receptors (proteins, antibodies, enzymes) and ligands (small molecules, antigens, drug compounds). FAC-MS is able to locate and characterize key molecular recognitions in complex biological systems that are otherwise "hidden" without the requirements of labeling. Currently, no other alternative method can provide unbiased, direct measures of binding affinity in a rapid and easy to perform manner as FAC-MS. Thus, it will allow for rational design and development in biological detection & identification, and medical countermeasure currently of interest to the CF and DRDC.

Protection against CB agents requires rapid developments and screening of new toxin antagonists and/or new vaccines. Better understanding of how CB threats work in humans to cause harm is important to provide knowledge for medical countermeasure developments. Moreover, identification of binding receptors for CB agents can be used as recognition sites for CB detection and identification. The areas that will benefit from the FAC-MS technology includes: vaccine development, botulinum neurotoxin antagonist research, and detection & identification of cell surface receptors for CB agents.

Vaccine development requires understanding in specific interaction between antibody and antigen (i.e. antigenic epitope mapping). In recent studies (1, 2), the structural spike (S) protein of severe acute respiratory syndrome coronavirus (SARS-CoV) was used as a target for the development of immunity-based prophylactic, therapeutic, and diagnostic clinical techniques against SARS-CoV. Once fragments of the S protein were produced and used in immunization, repeated ELISA and western blots were used to confirm production of antisera and/or monoclonal antibodies. Systematic mapping requires more ELISA and western blot experiments to identify fragments that were antigenic epitopes. FAC-MS can streamline this process by reducing the requirement for reagents and time-consuming experimentations.

Moreover, FAC-MS can be used to identify small molecules that will block the entry of SARS-CoV (3).

Botulinum neurotoxin (BoNT) is considered the most poisonous substance because of the extremely low dosage required for toxicity (intravenous LD_{50} is 1ng/kg). The toxic nature of BoNT makes it a potential candidate for use as biological weapon. BoNT contains a light chain (LC, 50kDa) and a heavy chain (HC, 100kDa) that are held together by a disulphide bond. The HC is responsible for binding recognition to the surface of nerve cells through an unknown cell surface receptor. Upon binding, the toxin internalizes into the nerve cell by the means of receptor-mediated endocytosis. The LC is a zinc-dependent endopeptidase that acts as a proteolytic enzyme on the substrate peptide of known structure. The proteolytic activity is required for toxicity. DRDC Suffield has many successful research projects done on BoNT (4, 5) and is currently engaged in more areas to further the success, such as protein fingerprinting of BoNT by capillary electrophoresis with laser-induced fluorescence detection, and development of cell-based assays and mouse test models for medical countermeasures. FAC-MS can aid in areas such as high throughput screening of potential drug molecules, and detection and identification of cell surface binding receptor for BoNT.

Although sulphur mustard (HD) has been considered a chemical weapon for over 80 years; there is no antidote, nor a clear understanding of its biochemical mechanism of action (6). Recent studies by Sawyer et al. suggested L-thiocitrulline (L-TC) has a prophylactic affect on the toxicity of HD in human keratinocytes, and its fast action (1 min prior to HD exposure) indicates that L-TC may bind to a cell surface structure (7). This hypothesis can be tested using FAC-MS and immobilized L-TC as “fishing bait” to identify interacting biomolecule(s) from keratinocyte membrane extracts. Multiple iterations of the affinity pull-down analyses provide a powerful, fast proteomic approach to identify and determine the biochemical pathway (protein cascade) involved in L-TC protection against HD toxicity in human keratinocytes.

Overview of FAC-MS

Frontal affinity chromatography is a separation technique that is used to study receptor-ligand binding interaction at equilibrium (8). Typically the ligand is continuously infused into a column containing an immobilized receptor, see Fig. 1 for a schematic of the FAC process. Any unbound molecules (blue dots in Fig. 1) will breakthrough at void volume (V_0), which accounts for the column and tubing volume between syringe and mass spectrometer. Interacting molecules (red dots in Fig. 1) will bind to the immobilized receptor on the column and once equilibrium is established, they will breakthrough at a later volume (V), thus differentiated from the unbound molecules.

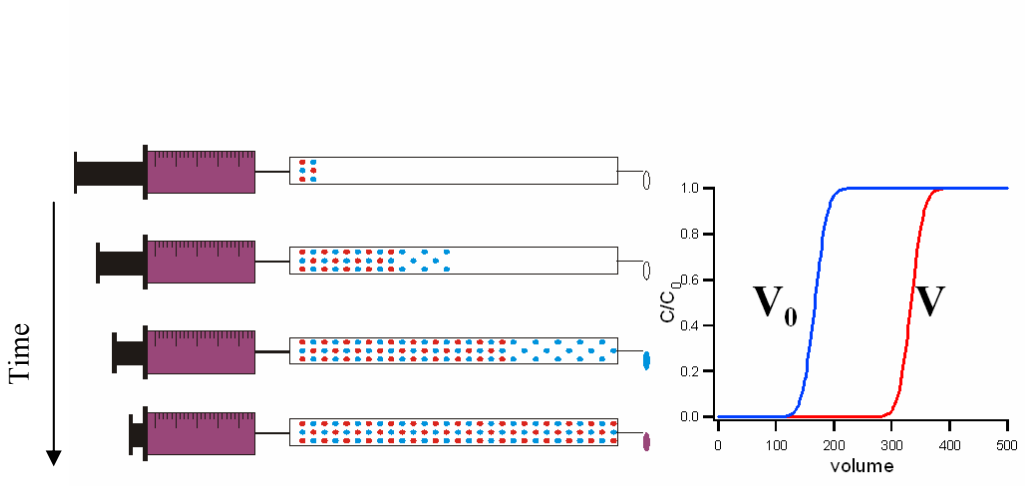

Figure 1. *Schematic of the FAC process. Sample is continuously infused at a predetermined flow through an affinity column over time (left). Breakthrough volumes for each solution component (indicated by the blue and red circles) are determined according to their interaction with the immobilized protein receptor. The breakthrough curves are shown on the right, where normalized concentration of analyte is plotted against breakthrough volume. The breakthrough curve of the non-binder or void marker (blue) exits earlier than that of the bound ligand (red). The difference between V and V_0 represents the breakthrough volume of the bound ligand.*

The equilibrium is described by equation 1, where R represents receptor, L represents ligand, and RL represents receptor-ligand complex. The dissociation constant, K_d, is a measure of the affinity of a receptor toward its ligand.

$$\mathrm{RL} \leftrightarrows \mathrm{R} + \mathrm{L} \qquad \text{where} \quad K_d = \frac{[R][L]}{[RL]} \qquad \text{Eq. 1}$$

K_d can be related to chromatographic parameters where B_t is the total number of immobilized receptors, $[A]_0$ is the initial concentration of ligand, V_i is ligand elution (or breakthrough) volume, and V_0 is void marker breakthrough volume. Equation 2 is analogous to the Michaelis-Menten equation in enzyme kinetic analysis. A rectangular hyperbola fitting to a graphical presentation of various ligand concentrations, $[A]_0$ against specific binding, $[A]_0(V_i-V_0)$ would yield K_d and B_t simultaneously. A linear direct proportion exists between the volume of sample required to achieve breakthrough (V_i-V_0) and the amount of immobilized receptor. Thus the lower the capacity of the FAC column, the lower the sample consumption.

$$K_d = \frac{B_t}{V_i - V_0} - [A]_0 \qquad \text{or} \qquad V_i - V_0 = \frac{B_t}{K_d + [A]_0} \qquad \text{Eq. 2}$$

FAC allows chromatographic separation based on the strength of the ligand-protein interaction. Ligand breakthrough occurs, at a fixed concentration, when an equilibrium is established between ligand and immobilized protein. The stronger the binding strength (lower K_d value), the longer time for ligand to reach equilibrium. This longer equilibrium time leads to a longer breakthrough time observed in the chromatogram. Thus, a ranking of molecules can be easily obtained in FAC experimental results (see Fig. 2). Historically, UV/Vis absorbance is employed for detection of ligand elution profile, therefore usually one ligand is analyzed at a time. Coupling mass spectrometry (MS) detection to FAC allows additional *m/z* ratio information, making FAC-MS amenable to multi-ligand binding analyses (9, 10). MS allows for a label-free assay, in which only one member of the interacting pair requires manipulation for the creation of the stationary phase. FAC-MS is similar to SPR technology in this regard, with the added benefit that a property of the ligand (i.e., its *m/z*) is used to generate binding data.

Figure 2 shows the results of a typical FAC-MS experiment. A total of 9 molecules (8 compounds of interest and a void marker) were monitored by MS. Each compound was infused at equivalent concentration, at 1mM through a column with immobilized sorbitol dehydrogenase. The K_d values of each of the 8 compounds of interest ranged from micromolar to nanomolar. The breakthrough curves showed void marker eluted first, then followed by compound 1 which has the weakest affinity toward sorbitol dehydrogenase, and the strongest binder (compound 8) was the last to breakthrough the column. The results clearly showed ranking of affinity toward sorbitol dehydrogeanse among the 8 compounds. Even in the case of isomers, compounds 3, 4 and 5, with identical m/z ratio can be differentiated by their affinity towards sorbitol dehydrogenase. Using the MS/MS capability to monitor fragmentation patterns of compounds 3, 4, and 5 (the magenta trace), each compound can be monitored separately (data not shown). It was identified that one of the 3 compounds had stronger affinity toward sorbitol dehydrogenase than the other two. Another typical observation was the normal breakthrough curve for void marker and the strongest binder (compound 8) but a transient overconcentration in all other compounds.

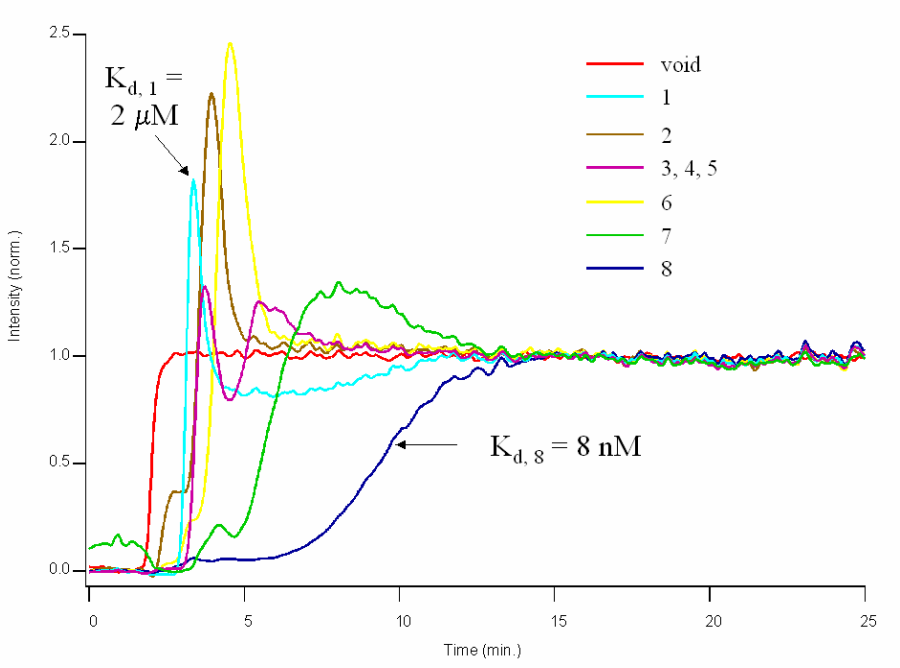

Figure 2. *FAC-MS experiment ranks ligands. A collection of 8 interacting compounds and a void marker, each at a concentration of 1mM, were infused on a sorbitol dehydrogenase column. Their Kd values span the range of micromolar to nanomolar. The results clearly show ranking of compounds even among isomers (compounds 3, 4, and 5) detected by mass spectrometer.*

The transient overconcentration of compounds 1-7, as shown in Fig. 2, is due to a competition between all interacting molecules for the binding site on the immobilized receptor. This transient phenomenon is a displacement of a bound ligand by a stronger ligand toward the immobilized receptor. Figure 3 depicts a short section of the column containing immobilized protein. Initially, the weak ligand propagates through the column faster and binds to the immobilized receptor. After a short time, the strong binder, propagates to the same section of the column, will bind to the same immobilized receptor and displaces the bound weak ligand. Therefore, one observes the transient overconcentration of the weak ligand. Once equilibrium is achieved, then the concentration of the weak ligand will fall back to its original concentration. Thus the competition among all 8 interacting compounds resulted in multiple displacement observed in Fig. 2. The strongest ligand (compound 8) did not show any roll-up because there wasn't any ligand to displace it. Neither did void marker show any roll-up because it did not interacted with the immobilized protein.

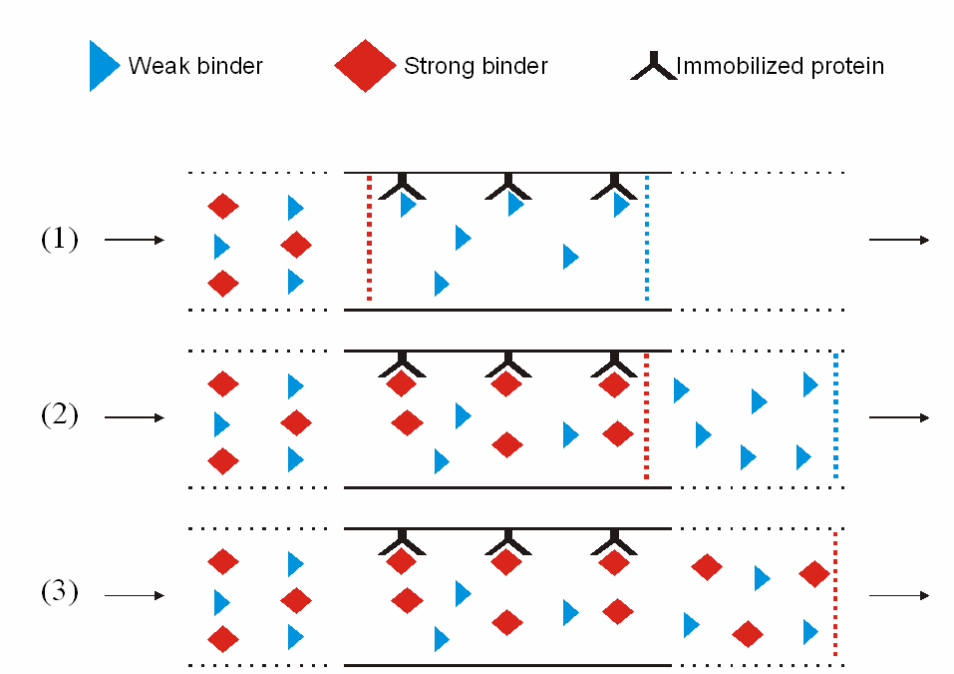

Figure 3. *A section of affinity column shows displacement of weak ligand by a strong ligand on a column containing immobilized receptor. The weak binder can propagate faster through the column and binds to the immobilized receptor (1). Subsequently the weak binder is displaced by the strong binder and a transient overconcentration (2) occurs in the liquid phase. Once equilibrium is established (3), the concentration of the weak binder falls back to the initial level.*

FAC-MS experimental setup involves simple fluidics as shown in Fig. 4. Syringe pumps are used to drive three independent syringes, two syringes are connected to a FAC column through a fluid switching valve. One syringe contains the running buffer for conditioning the protein receptor column, while the other contains analytes in the same running buffer. The fluid switching valve will change from buffer to analyte solution when the column is conditioned. The eluent from the column is subsequently mixed with an organic makeup flow fluid prior to introduction to the electrospray ionization mass spectrometer (ESI MS). The columns are bare fused silica capillaries with dimensions typically of 2.5cm long, and with 360µm o.d. and 250µm i.d. The column volume is typically about 500µL. Packing materials are controlled pore glass (CPG) coated with streptavidin. Biotin-tagged proteins are bound to CPG via the strong and specific interaction between streptavidin and biotin ($K_d = 10^{-14}$M). The protein is strongly attached to CPG and can be used repeatedly, where in some cases a single column can be used over a period of 3 months (data not shown).

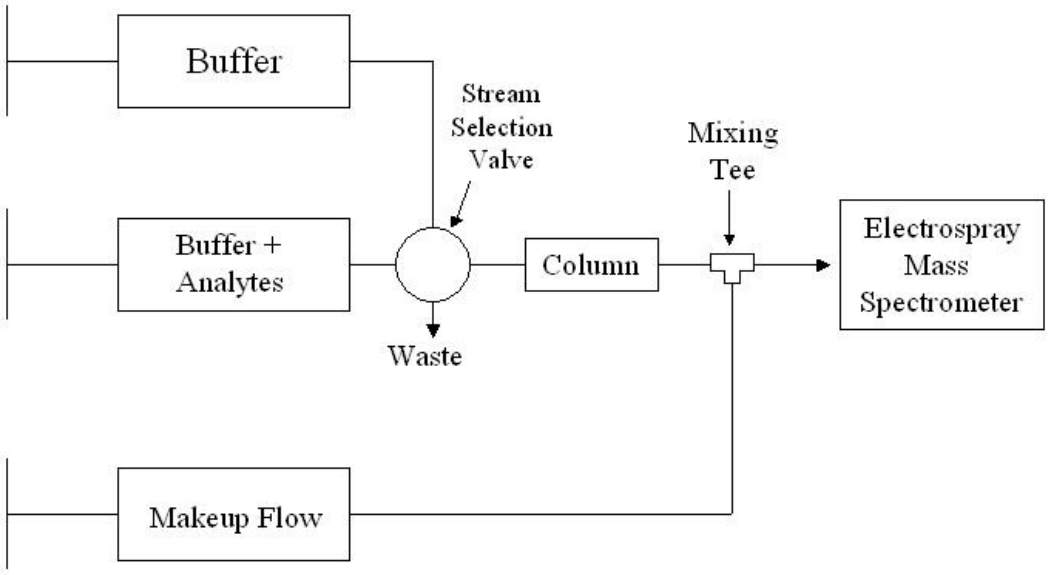

Figure 4. *Schematic of the FAC-MS system for monitoring molecular interactions. Syringes containing either buffer or buffer containing analytes (ligands) are connected to the FAC column through a stream selection valve. Makeup flow, typically a volatile, organic solvent, is introduced immediately postcolumn through a mixing tee, with the combined flow introduced into an electrospray mass spectrometer.*

Mass spectrometry is an important technique for CB analysis and for the study of ion chemistry. There are 5 components in a MS: sample introduction, ionization, mass analyzer, ion detector, and a computer data system. The ionization component, mass analyzer and ion detector are operated under vacuum. Work under vacuum is required to reduce collisional defocusing (or scattering), electrical discharge and high background from air, resulting in high sensitivity and resolution in detection. The ionization methods are for creating ions in the gas phase. Soft (non-destructive) ionization methods such as electrospray (ESI) and matrix-assisted laser desorption (MALDI) are popular choices for analysis of biological samples. In ESI, ionization is done at atmospheric pressure. The mass analyzer functions to separate ions according to their mass-to-charge (m/z) ratio, and to maximize the resolved ion intensity. The ion detector (eg. electron multiplier detector) is to detect the individual ions after the mass analyzer and convert to readable data on mass spectra (ion intensity vs. m/z).

In FAC-MS, sample is introduced from a stream of fluid eluting from the affinity column, and ionized in the ESI interface. ESI is an important interface for FAC-MS because it allows online detection of column eluent without decoupling the chromatography from the MS. The electrospray phenomenon was first described by Zeleny in 1917 (11), although the first combined ESI-MS results were reported in 1984 (12). The electrospray mechanism (13) involves four major processes: production of charged droplets at the ESI capillary tip, then several cycles of shrinkage of charged ESI droplets and repeated droplet disintegrations, and finally generation of gas phase ions (Fig. 5). The fluid eluting from the affinity column flows continuously through a capillary. The capillary is charged with a high voltage of typically 2-5kV, forming a Taylor cone at the tip of the capillary. A liquid filament forms at the tip of the Taylor cone, and separate charged droplets form at the end of the filament jet. The charged droplets undergo repeated evaporation and droplet disintegration to form gaseous ions prior to entering the mass analyzer.

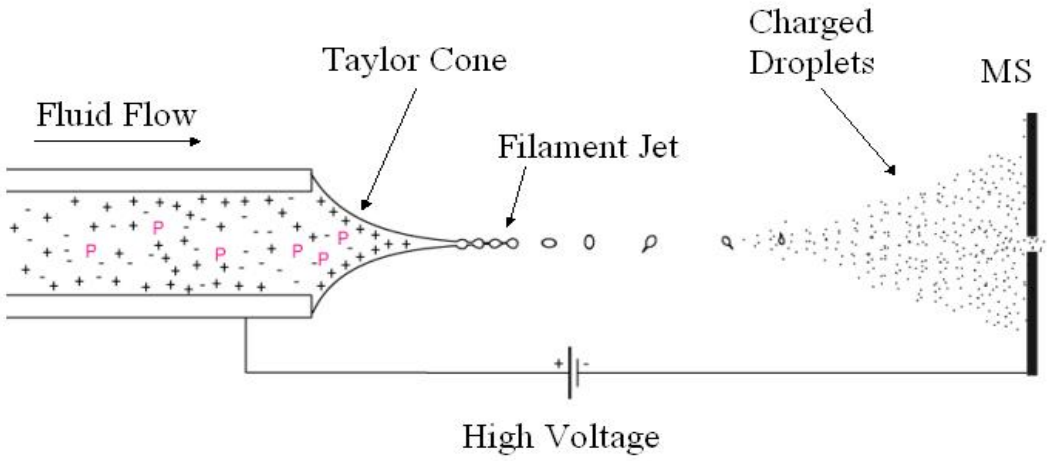

Figure 5. *Schematic of the electrospray ionization. Fluid, containing analytes such as peptides or proteins (P), flows from a capillary charged with high voltage. At the capillary tip, build up of charges at the liquid surface will cause a Taylor cone to form. At high electrical fields, the cone becomes unstable and a liquid filament forms. Downstream of the filament jet, charged droplets form. Repeated cycles of droplet shrinkage and disintegration result in formation of gas phase ions prior to entering the MS.*

Use of FAC-MS for :

Rapid determination of binding affinity between antibody and antigen pairs

The first article to describe use of FAC-MS is for the study of epitope mapping of abequose saccharide on the O-antigen of *Salmonella paratyphi B* with a monoclonal antibody immobilized on the affinity column (14). Therefore, the idea of using FAC-MS for rapid determination of binding affinity between recombinant antibody and antigen pairs just becomes natural. In the report, Schriemer et al. immobilized an antibody onto a column, and applied a solution containing six oligosaccharides to the column, and obtained elution profiles for each oligosccharides. Three oligosaccharides appeared at the breakthrough volume, but the other three gave different elution volumes, and consequently different dissociation constants. The ability to obtain dissociation contants for multiple analytes in a single experiment is powerful since such application is not easy for other binding assays such as ELISA or SPR.

The application in this area is unlimited. In cases such as vaccine development, binding domains can be mapped for optimization of vaccines, and any recombinant vaccine developed can be tested against the antibody in the same assay. For cases where there are different domains, example of protective antigen (*Bacillus anthracis*) having four domains, the binding events can be monitored and screened separately for antibody against each domain for specificity. Using FAC-MS, will minimize the use of reagents (such as secondary antibody required for ELISA detection) and thus reduce costs.

Screen and rank toxin antagonists

Development of FAC-MS has primarily focussed on drug discovery for the pharmaceutical industry because of its power in handling large mixtures of analytes and ability to rank and differentiate the binding strength of each analyte, even if some analytes were detected as isomers (see Fig. 2).

The application of FAC-MS in high throughput screening and ranking of inhibitors has exploded to include proteins such as epidermal growth factor receptor (15), anti-galactose antibody (16), hepatitis C virus protease (17), β-galactosidase (9), sorbitol dehydrogenase (10), polypore mushroom lectin (18), human estrogen receptor β (10), erythropoietin-producing hepatocellular B2 (EphB2) receptor (19), and a cell surface receptor for SARS-CoV (3). Therefore, FAC-MS will be a great tool to screen for antagonists against toxin, such as BoNTs, tetanus toxin, ricin, etc.

New methods in drug discovery based on combinatorial chemistry, automated peptide synthesis and computer modeling can produce very large numbers of potential small molecule and peptidic drug candidates (so-called libraries). To make use of the libraries and to identify active compounds, novel methods in rapid drug screening are required. Previous work was carried out to investigate activity-based bioassays to screen potential inhibitors against BoNT LC using high performance liquid chromatography (HPLC) or capillary electrophoresis (CE) separation

techniques (4, 5). The peptide libraries consist of capped tetrapeptides where the second peptide is β-amino butyric acid for increased flexibility of the peptide structure, thus increasing the opportunity of side chain interactions for molecular and conformational diversity (20) within a relatively small library set (12^3 or 1728 tetrapeptides). The peptides are capped with acetyl and amide groups at the N- and C-termini, respectively. Preliminary results in HPLC and CE assays (4, 5) showed a few promising candidates for further investigation. The publications reported BoNT serotype A was tested against libraries containing mixtures of 12 compounds and required further deconvolutions to obtain results for each individual tetrapeptide. FAC-MS can provide an accurate measure of binding affinities between the BoNT and its antagonists, rank-ordering capability to evaluate libraries containing a large number of compounds, and provides inhibitor identification by MS detection in a single experiment. The speed and wealth of information generated by FAC-MS can be complementary to the activity-based bioassays. All of the information is crucial in order to understand the mechanism of binding which is the first step in developing better medical countermeasures.

Proteomic approach to determine unknown cell surface receptor to CB agents

Since the success of sequencing the human genome, the interest in proteomic research has caught on to use analytical techniques and computational database search capabilities to understand protein interactions. Proteins are often associated with diseases, thus proteomic approaches identify novel proteins involved in key biological processes in the cell may serve as protential drug targets. For drug discovery, proteomics is an important area of research so drugs can be "tailor-made" with more specificity and fewer side affects. There are reports of proteomic approach to development of vaccines and drugs against *Bacillus anthracis* infection (21), and discovery of small molecule blocking entry of SARS coronavirus into host cells (3).

There are two specific receptors that are of interests, the receptor(s) binding BoNTs, and the keratinocyte cell surface receptor binding L-thiocitrulline (L-TC) triggering prophylactic protection against sulphur mustard (HD). In the case of identifying the BoNT receptor, it not only is beneficial to drug discovery, it can also be used for bio-detection, similar to reports on using glycosphingolipids as receptor molecules for detection of cholera toxin, ricin, diphtheria toxin, staphylococcal enterotoxin B (SEB), BoNTs, and tetanus toxin (22-28). In the case of understanding protein cascade associated with L-TC protection, better design of the drug candidate can be done to improve its prophylactic effects.

The most common functional proteomic method to identify unknown cell surface receptor is a sequential process of tagging a molecule of interest (protein or small molecule), affinity pull-down, SDS-PAGE protein separation, protease digestion, then tandem mass spectrometric analysis (see Fig. 5 for the schematic). First, a tag is attached to the molecule of interest to create a bait molecule. The bait is then exposed to cell membrane lysate and allowed to interact with proteins within the lysate to form a protein complex. Then, the bait-complex is pulled out with an affinity column, the interacting proteins are then eluted and separated on SDS-PAGE. Each protein band is then excised and digested by specific protease, and identified using standard mass spectrometry methods. Many iterations using different protein molecules as baits will subsequently provide an insight to the protein cascade, or map, involved in the particular pathway (29-31).

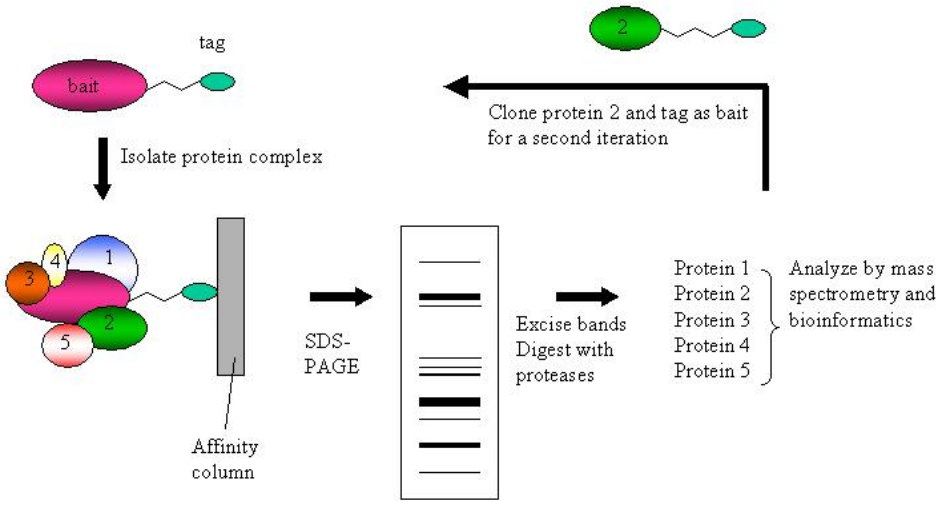

Figure 6. *Schematic of a functional proteomic approach to map protein cascades. The approach includes using in vitro affinity pull-down, separation of proteins by SDS-PAGE, and anlysis by MS/MS and bioinformatics. A protein or a small molecule can be used as a bait to "fish" for any interacting proteins. The complexes are separated from crude cell lysate by affinity chromatography that recognizes the tag on the bait molecule. Then, protein complexes are separated on gel, protein bands are excised and individually digested with proteases into peptide fragments. Identity of each protein can be determined by tandem mass spectrometric analysis. One of the interacting proteins identified from the complexes can be used as bait for a second iteration. After several iterations, the protein cascade due to the first bait molecule can be determined.*

FAC-MS can streamline this combination of affinity pull-down, SDS-PAGE, MS sequencing process to monitor both strong and weak binders. Bait protein can be immobilized onto FAC column, cell lysate can be infused through bait column and eluent can be monitored for any interactions, ascending from weak to strong. The identification of each interacting protein can be done with MS/MS analysis. Every subsequent iterations will involve immobilization of an interacting protein identified from the previous FAC-MS analysis. After several iterations, a protein interaction map involved in the cascade can be determined. In addition, FAC-MS can be used to validate any protein target pulled out from this process. Recent reports by Toledo-Sherman et al. (32-33) studied the mechanism of action of methotrexate, a clinical agent used in cancer, immuosuppression, rheumatoid arthritis, and other highly proliferative diseases. The authors used the above-described process and confirmed one of the protein targets identified, hypoxanthine-guanine amidophosphoribosyltransferase, had a K_d of 4.2μM toward methotrexate. These data not only shed light into the mechanism of action of methotrexate in oncology and other diseases, they may also help explain some effects unaccounted for due to this drug.

Conclusion

In order to provide effective medical countermeasures and biological detection & identification technologies to the CF, a better understanding of how CB threats work in humans to cause harm is important. Molecular interactions are fundamental to the understanding of biochemical pathways to further advance developments in medical countermeasure and detection/identification technologies. Current analytical technologies are not amenable to both high-throughput analysis and the provision of high quality information on molecular recognitions and interactions. FAC-MS can provide rapid, unbiased knowledge in binding interaction between receptor and its ligand(s) without the requirements of labelling. It can measure binding strength at true equilibrium conditions. FAC-MS is amenable to high-throughput screening due to its intrinsic ability to separate bound versus unbound molecules with the addition of molecular weight identification, thus it is useful in the drug discovery and optimization process, and in identification of new protein targets against CB agents for both new strategies in biological detection/identification and medical countermeasure. This capability provides a good foundation for rational designs in new drugs and detection/identification methods against CB agents.

The proteomic approach to determine unknown cell surface receptor to CB agents using FAC-MS is an essential part of a project arrangement in proteomics method development among Canada, the Netherlands, and Sweden under the CA/NL/SE Cooperative Science and Technology Memorandum of Understanding (MOU).

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List of symbols/abbreviations/acronyms/initialisms

$[A]_0$	Initial Ligand Concentration
BoNTs	Botulinum neurotoxins
B_t	Total Binding Sites
CB	Chemical Biological
CE	Capillary electrophoresis
CF	Canadian Forces
CPG	Controlled pore glass
DNA	Deoxy-nucleic acid
DND	Department of National Defence
DRDC	Defence Research & Development Canada
ELISA	Enzyme-Linked Immuno-Sorbent Assay
EphB2	Erythropoietin-producing hepatocellular B2
ESI	Electrospray Ionization
FAC-MS	Frontal Affinity Chromatography with Mass Spectrometry
HC	Heavy chain
HD	Sulphur mustard
HPLC	High performance liquid chromatography
i.d.	Inner diameter
K_d	Dissociation Constant
kDa	Kilodalton
L	Ligand

LC	Light chain
LD_{50}	Lethal dose causing death of 50%
L-TC	L-thiocitrulline
m/z	Mass-to-charge
MALDI	Matrix-assisted laser desorption ionization
MOU	Memorandum of understanding
MS/MS	Tandem mass spectrometry
o.d.	Outer diameter
R	Receptor
RL	Receptor-ligand complex
S	Spike
SARS-CoV	Severe acute respiratory syndrome coronavirus
SDS-PAGE	Sodium dodecyl (lauryl) sulphate – polyacrylamide gel
SEB	Staphylococcal enterotoxin B
SPR	Surface Plasmon Resonance
UV/Vis	Ultraviolet and visible light
V_0	Void Volume
V_i	Ligand Elution (or Breakthrough) Volume

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(U) Frontal affinity chromatography with mass spectrometry detection (FAC-MS) is a powerful technique for the study of molecular interactions between receptors (proteins, antibodies, enzymes) and ligands (small molecules, antigens, drug compounds). Such interactions are fundamental to biochemical pathways, from the detection of a protein cascade involved in a disease state, to the study of the disease mechanisms, to high-throughput screening for the identification and optimization of lead compounds for drug discovery. FAC-MS is able to locate and characterize key molecular recognitions in complex biological systems that are otherwise "hidden" from current technologies. Thus, this cutting-edge technique can steer biotechnological research through key areas and eliminate many years of "hit-and-miss" experimentation. It will allow for rational design and development in areas, such as bio-detection and identification, and medical countermeasure, which are currently of interest to the CF and DRDC. FAC-MS is amenable to high-throughput screening of large collections of compounds simultaneously. Currently, no other alternative method can provide unbiased, direct measures of binding affinity in a rapid and easy to perform manner as FAC-MS. This article outlines the plan of adapting FAC-MS from industry (Protana Inc.) to DRDC Suffield for defence research incorporating toxin research, drug discovery, recombinant antigen-antibody optimization and proteomics.

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