Frontal affinity chromatography with MS detection (FAC–MS) in drug discovery

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The emergence of a relatively new technique resulting from a combination of frontal affinity chromatography coupled with MS detection (FAC–MS) has extended the capabilities of MS in drug discovery and development. Its application in a broad range of biological systems, together with its label-free operation, relatively high throughput, ability to rank ligands and determine K_d, makes FAC–MS a universal tool enabling convenient and efficient screening in the identification of new potential drug leads. Here we will highlight FAC–MS screening studies and discuss where it can be applied in evaluating multiple protein-binding sites, protein–protein interactions and inactive proteins, and also in determining selectivity.

In recent years, the use of MS has become an indispensable tool in the pharmaceutical industry. Although its main use has been at the drug development stage, it is now being identified as a useful detection method in the drug discovery process [1–3]. Within this process, the pharmaceutical industry has relied on assaying large historical compound libraries for the identification of small-molecule hits against therapeutic targets. During the past decade, advances in HTS technologies, combinatorial chemistry and robotics have dramatically transformed the hit identification process. However, despite the fact that HTS of large compound libraries does indeed provide hits (albeit at a much lower rate than expected), there are numerous difficulties associated with HTS assay development that are being encountered [4], such as miniaturization costs, sticky or poorly soluble compounds, liquid dispensing and protein production. Furthermore, the majority of these HTS assays involve detection methods such as fluorescence, radioactivity or absorbance, which, although useful, only provide indirect evidence of binding [5]. This is because the detection of the binding event is not based on a distinct feature of one of the binding components. Therefore, a screening system that is a measure of direct binding, even at a moderate throughput, would be advantageous. There are only a few technologies that provide binding data and species identification [6] but the emergence of a relatively new technique based on a combination of frontal affinity chromatography with MS detection (FAC–MS) has been shown to be of value in the screening of mixtures and the determination of dissociation constants (K_d) and has the potential to become a useful method in modern drug discovery.

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Principles of FAC–MS

FAC–MS was first introduced by Schriemer in 1998 [7], and is based on the continuous infusion of ligands over a protein target immobilized onto a solid support column, with the eluting ligands detected by MS. In contrast to traditional 'capture and release' affinity chromatography methods, FAC–MS is based on an ongoing equilibrium between ligands flowing through the column containing the immobilized protein target. As ligands flow through the column

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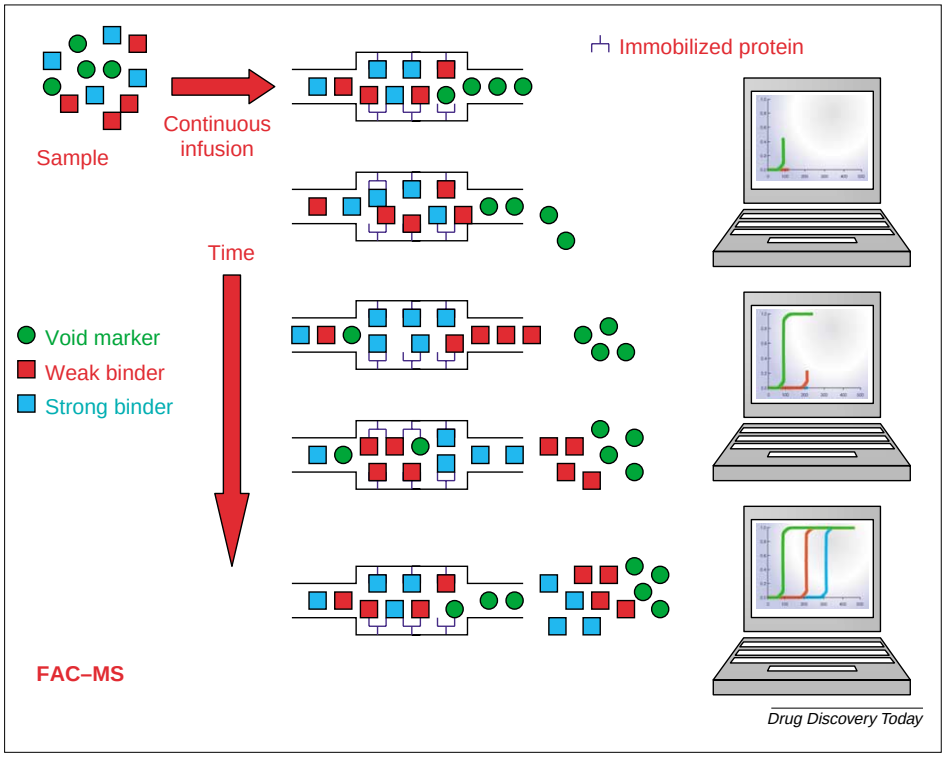

FIGURE 1

The principle of FAC–MS is depicted, whereby ligands and a void marker are continuously infused through a column containing an immobilized protein.

they might bind to the target with differing affinities. As a result, individual ligands are retained, based on their affinity in the column causing an increase in their 'breakthrough volume', that is, the effluent volume passing through the column that allows the output ligand concentration to equal the input ligand concentration. The breakthrough volume of the ligand, characterized as a sigmoidal front, is detected by MS by monitoring its m/z value and corresponds directly to the time that the ligand's front (breakthrough time) is observed to pass through the column (Figure 1). Therefore, depending on the affinity of the ligand for the target, FAC–MS offers a convenient way of measuring the relative binding strengths of ligands, which enables rapid ranking and ligand identification. With the use of a void marker – a compound that has no affinity for the immobilized protein target and gives the same elution front whether the target protein is present in the column or not – and MS detection, this technique enables multiple ligands to be evaluated simultaneously in a direct readout fashion by measuring the entire m/z range (so called Q1 scan).

The limiting factors with FAC–MS, as with most screening paradigms, involves DMSO concentration and compound solubility. To limit the amount of DMSO affecting protein stability the FAC–MS running buffer typically contains a maximum of 1–2% DMSO. Hence, if 5 mM compound stock solutions in DMSO are used, then 200 compounds can be screened per run (each at 0.5 μM) with a total DMSO concentration of 2%. If a particular protein target can tolerate an additional amount of DMSO then this number can increase accordingly. Nevertheless, with 200 compounds per run, and FAC–MS runs typically averaging 30 min, with continuous operation the number of compounds screened approaches 10,000 in a 24 h period.

The concept of using an 'indicator' for monitoring real-time competition with ligands in screening applications has increased the capability of FAC–MS [7]. An indicator is a compound detectable by MS that binds to a specific site on the immobilized protein target with a known affinity in a specific column infusion time. In these screening cases using an indicator, only the indicator and void marker are monitored by MS. Although there is no set affinity requirement for an indicator to an immobilized target, for practical reasons the affinity should be in the 0.5–50 μM range. Therefore, these two FAC–MS screening possibilities – with and without an indicator – give FAC–MS extra flexibility in screening assays and are suitable for the identification of weak and tight binders (with K_d ranging from pM to ~0.5 mM).

It has been shown that in the development of FAC–MS, using frontal affinity chromatography theory [8–12], the dissociation constant, K_d, can be related to the FAC–MS chromatographic experimental parameters [12] via Equation 1:

$$K_d = \frac{[A][P]}{[AP]} = \frac{[A]_0\{[P]_0 - [A]_0(V - V_0)/v}{[A]_0(V - V_0)/v} = \frac{B_t}{V - V_0} - [A]_0$$

where $[A]_0$ is the ligand infusion concentration, $[P]_0$ is the amount of immobilized protein per unit column volume, v is the total bed volume of the column, B_t is the total amount of immobilized protein equal to $v[P]_0$, V is the elution volume of ligand, A, V_0 is the elution volume of A in the absence of immobilized protein and $[A]_0(V - V_0)$ corresponds to the amount of A specifically adsorbed to the immobilized protein. Equation 1 can then be rearranged to the more useful Equation 2:

$$V - V_0 = \frac{B_t}{[A]_0 + K_d}$$

The use of Equation 2 enables the determination of K_d using either a modified staircase approach or an indirect method using an indicator. The modified staircase approach is accomplished by infusing a FAC–MS column sequentially starting with the lowest of a series of concentrations of a ligand, A. Hence, for each concentration the breakthrough volume ($V-V_0$) is determined by monitoring the ligand and

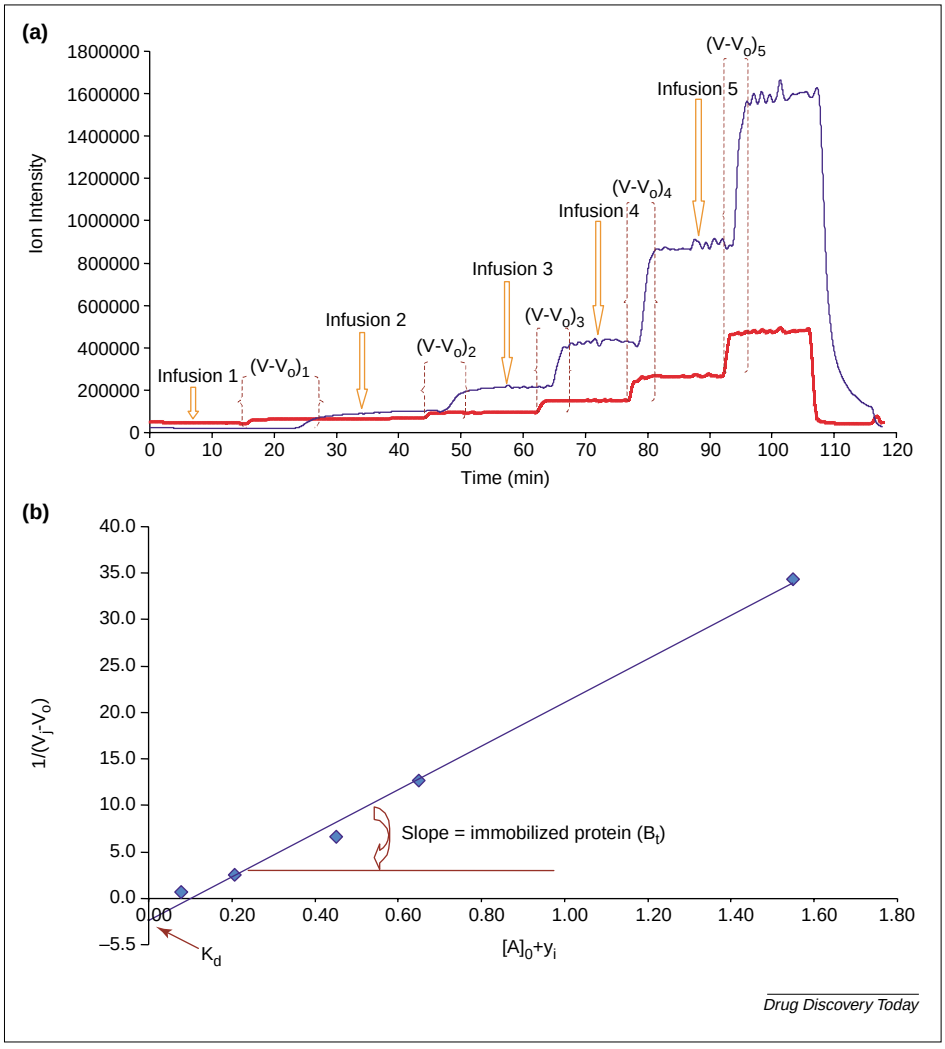

FIGURE 2

Depiction of a typical 'modified staircase' experiment to evaluate K_d for a protein target (immobilized in the column) and a small molecule ligand. (a) The ligand (blue) is infused at increasing concentrations starting from an initial (*i*) to a final (*j*) concentration along with a void marker (red). **(b)** The reciprocal of the breakthrough volumes, $1/(V_j\text{-}V_0)$, are plotted against the summed ligand concentrations, $[A]_0 + y_j$ according to the equation [17], to produce a linear correlation from which the K_d value can be determined from the y intercept. The total amount of immobilized protein (B_t) in the column is then obtained from the slope.

void marker by MS and a plot of the summed ligand concentration versus the reciprocal of $V\text{-}V_0$ generates a line with the y intercept equal to $-K_d$ and the slope equal to B_t (Figure 2). For this modified staircase approach, the summed concentrations refer to the initial concentration of the ligand for the first step of the staircase but for the second step of the staircase, it will be the sum of the initial concentration plus the concentration of the second step. Similarly, the concentration of the ligand for the third step of the staircase will be the sum of the initial, second and third steps, and so on for the remaining concentrations.

For cases where MS cannot detect the ligand, or if it has a strong affinity for the immobilized protein, the indirect method must be used. In this case, a FAC–MS column is again sequentially equilibrated with the lowest of a series of concentrations of a ligand; however, in between each of the ligand equilibrations, the indicator, void marker and the ligand (at the appropriate concentration) are infused and $V\text{-}V_0$ for the indicator is measured. With this indirect method, the ligand competes for binding sites with the indicator and as the concentration of the ligand increases, $V\text{-}V_0$ for the indicator decreases.

Screening applications

Since 1998, several screening applications have appeared that demonstrate the capability of FAC–MS to handle a broad range of biological targets.

Antibody based targets

The first application by Schriemer used the monoclonal carbohydrate-binding antibody that recognized the 3,6-dideoxy-D-xylohexose (abequose) epitope [7]. Since then, there have been other antibody-related targets immobilized in FAC–MS applications, specifically a polyclonal antibody prepared using a known anti-epidermal growth factor receptor (anti-EGFR) inhibitor coupled to BSA [13] and human anti-Gal IgG antibodies [14]. A polyclonal antibody raised to mimic the active site of hepatitis C virus protease was used to screen an extract of *Phyllanthus urinaria L.* with a set of five natural products from this extract found to have high inhibitory activity [15].

β-galactosidase

One of the first screening applications of FAC–MS involved the use of immobilized β-galactosidase [16]. A library of 89 modified β-galactopyranosides, each representing 4 diastereomers for a total of 356 library members, were grouped into mixtures of 24–40 compounds and analyzed by FAC–MS. The subsequent deconvolution procedure led to the identification of 34 compounds with K_d values less than 10 μM. This study with immobilized β-galactosidase was also the first to describe the 'roll-up' phenomenon (Figure 3). If there is a ligand in the screening mixture that has an affinity for the immobilized protein target that is greater than the affinity of the indicator then this ligand will, before equilibrium is established, transiently displace more of the indicator, thus generating an over concentration (or peak like shape) of the indicator in the chromatogram. By contrast, if the screening mixtures only contain ligands that have weaker affinities for the target than the indicator, then depending on the degree of affinity, only the indicator will elute earlier (i.e. its front will shift to the left) and there will be no roll-up.

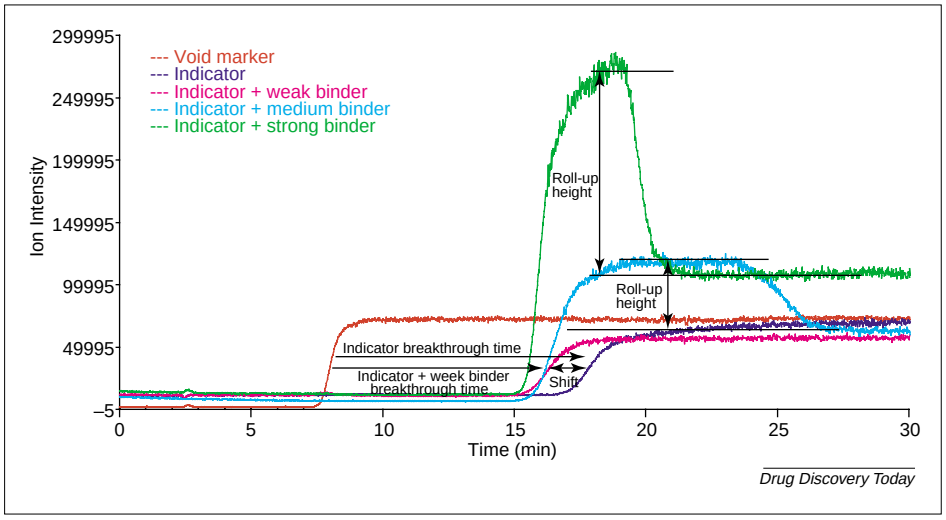

FIGURE 3

FAC–MS indicator responses (roll-ups and shifts) typically seen in screening experiments. The breakthrough front of the indicator (blue trace) is shown in the absence of any competing ligands. A ligand that is competitive with the indicator but has a K_d that is weaker than the K_d of the indicator will generate a shift or the indicator's front to the left (magenta trace versus blue trace). A competitive ligand with a K_d that is comparable to, or slightly stronger than, that of the indicator will generate a roll-up effect (a transient over-concentration of the indicator) that dominates over the shift of the indicator (turquoise trace). Competitive ligands that are much stronger binders than the indicator will generate substantial roll-ups of the indicator (green trace).

Sorbitol dehydrogenase (SDH)

Using ligands with varying degrees of affinity for SDH [17] – ranging from 0.12 to 3.3 μM – FAC–MS was able to rank the compounds based on their breakthrough curves. The relative breakthrough volumes of the compounds correlated well with their IC_{50} values.

Mushroom lectin

Immobilized polypore mushroom (*Polyporus squamosus*) lectin (PSL) was used to screen a variety (22) of sialylated or sulfated oligosaccharide mixtures [18]. Sialylated oligosaccharides are crucial determinants in viral and bacteria adhesion and PSL recognizes sialylated oligosaccharides. The binding affinity and specificity of PSL was examined by FAC–MS using these oligosaccharide mixtures.

Human estrogen receptor β (hERβ)

An immobilized 21 amino acid peptide of hERβ ligand binding domain tagged with 6 histidine residues (a 'His tag'), and three cysteine-to-serine mutations, was used in FAC–MS studies to determine binding affinities of several known ER ligands. This expressed peptide had been shown to stabilize ERβ but not affect ligand-binding activity [19]. The K_d values of the various ER ligands that were determined by FAC–MS were comparable to literature values [17].

EphB2 receptor tyrosine kinase

The erythropoietin-producing hepatocellular B2 (EphB2) receptor tyrosine kinase was immobilized and a series of commercially available kinase inhibitors were profiled by FAC–MS, using the ligand WHI-P180 as the indicator and ranked using the '% shift' of the indicator [20]. As exemplified in Figure 3, the breakthrough time for the indicator is first measured alone, then in the presence of ligands. To compare the reductions in the breakthrough times for indicator in the presence of the ligands, the % shift is quantified from Equation 3,

$$\%\,Shift = (t_{I-t})/(t_I - t_{NSB}) \times 100\% \quad (3)$$

where t is the breakthrough time difference (measured at the inflection point of the sigmoidal front) between the indicator and void marker in the presence of the ligand, t_{NSB} is the non-specific binding breakthrough time difference between the indicator and void marker in the absence of immobilized protein or immobilized neutral protein (and is a constant for the indicator used) and t_I is the breakthrough time difference between the indicator and void marker in the absence of the ligand. In this manner, the % shifts can be used to rank the binding affinity of compounds for further analysis and, ultimately, further biological evaluation. In other words, the greater the % shifts, the greater the degree of competition for the indicator.

As with any binding assay, there is generally a concern regarding a correlation between the results of a binding assay and a functional activity measurement. For the case of EphB2, the FAC–MS % shifts of the kinase inhibitors not only correlated with K_d but also with the more traditional measurement of activity, IC_{50} values from an ELISA assay. As mentioned previously, similar correlations have been observed in FAC–MS studies for other immobilized proteins, namely SDH [17] and a polyclonal antibody acting as an epidermal growth factor receptor mimic [13]. Therefore, these correlations with IC_{50} indicate that FAC–MS can be used with confidence in practical screening applications with a variety of immobilized targets.

Differentiating between multiple binding sites

Protein targets often have multiple sites for potential inhibition, but these multiple sites are generally not specifically addressed using traditional screening methodologies. With the methods currently available, it is not usually possible, in an initial screen, to distinguish between the binding sites using traditional radioactive, luminescent or antibody-based assays [21]. Such screens are based on assays that are limited to measuring the functional effects of small-molecule interactions without revealing the site of inhibition. With these assays, once a ligand has been identified, additional structural studies are required to elucidate the site of binding. Protein kinases are an example

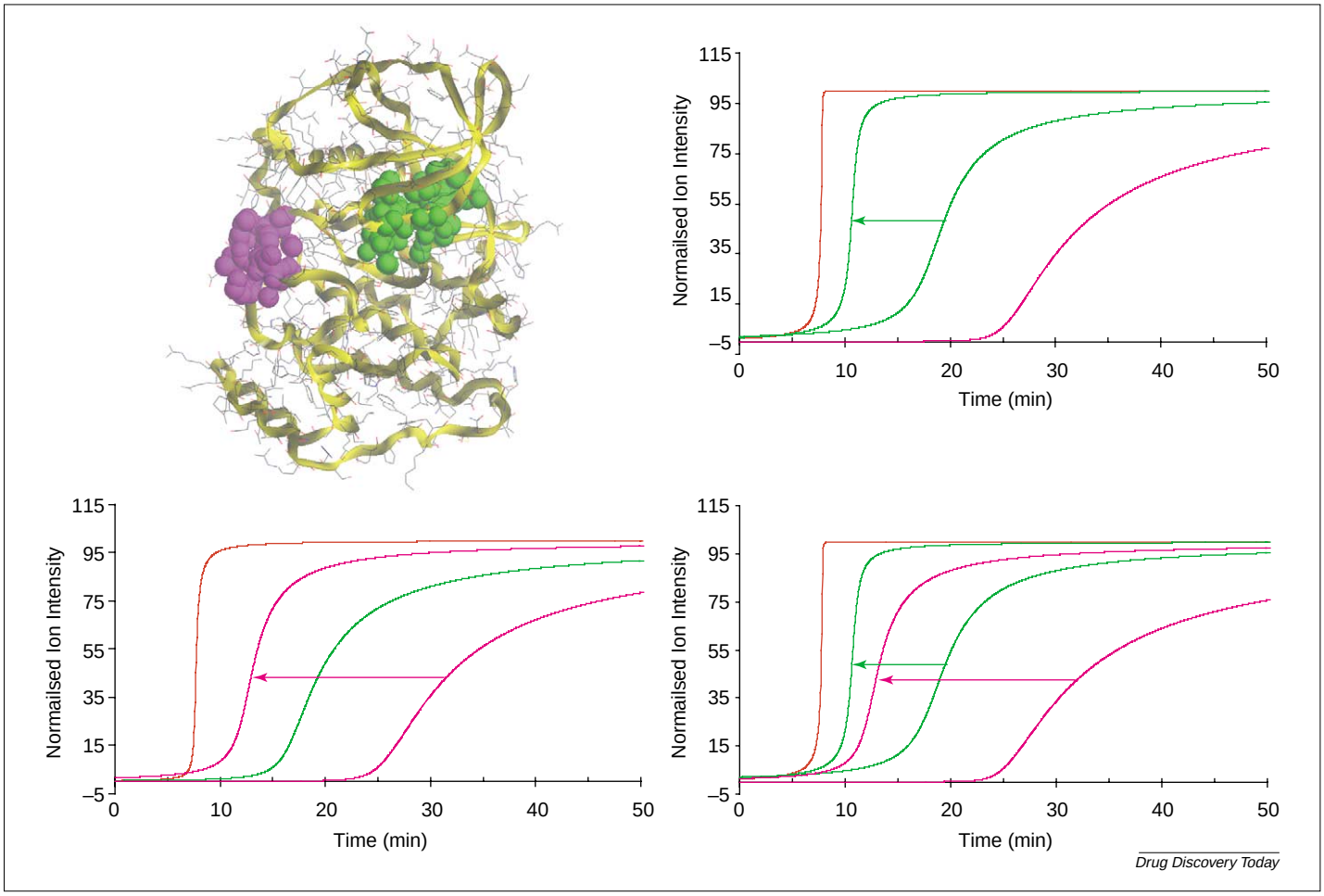

FIGURE 4

FAC–MS chromatograms of dual indicators for PKCα immobilized to CPG beads through the streptavidin–biotin complex. In the chromatograms, red lines correspond to the void marker, M3; magenta lines correspond to chelerythrine chloride (1 μM), the substrate site indicator and green lines correspond to PD153035 (1 μM), the ATP site indicator. Arrows indicate respective shifts. FAC-MS intensities normalized for clarity. (Lower left panel) The effect of adding WHI-P180 (5 μM) to the infusate. Only PD152025 (1 μM), the ATP site indicator, shifted to the left from 19 to 10 min. (Upper right panel) The effect of adding protein kinase C inhibitor peptide 19-36 (5 μM) to the infusate. Only chelerythrine chloride (1 μM), the substrate site indicator, shifted to the left from 30 to 12 min. (Lower right panel) The effect of adding both WHI-P180 (5 μM) and protein kinase C inhibitor peptide 19-36 (5 μM) to the infusate. Both indicators shifted to the same extent as when they were analyzed separately.

of therapeutic targets that possess multiple binding sites and because each protein kinase targets a specific protein substrate, it has been suggested that compounds that target the substrate-binding site, instead of the ATP-binding site, could provide drugs with improved kinase selectivity and potentially less toxicity [22].

An example of where FAC–MS has been used to evaluate different binding sites on the same protein has been shown using protein kinase Cα (PKCα) [23]. FAC–MS confirmation of PKCα activity was obtained by observing significant binding (delayed breakthrough time compared to the void marker) of both the substrate binding site and the ATP binding site ligands, chelerythrine chloride and PD153035, respectively. Neither compound affected the breakthrough time of the other compound, regardless of the concentration used. They were therefore able to act simultaneously and independently as dual indicators for their two respective binding sites of the catalytic domain of PKCα (Figure 4). With indicators for the two binding sites for PKCα established, when an ATP-binding-site ligand was added (WHI-P180), only the ATP-binding-site indicator shifted to the left and there was no effect on the substrate-binding-site indicator. An analogous situation occurred for the substrate-binding site, when a substrate-binding-site ligand (PKC inhibitor peptide 19–36) was added and only the substrate-binding-site indicator shifted. This suggests that screening with FAC–MS, in this fashion, would not only provide hit identification, but also binding site information in the same screen.

Inactive conformations

Nature has designed sophisticated processes to regulate kinase activity in cells [24]. Kinases themselves use a variety of autoinhibitory mechanisms to maintain inactive configurations in quiescent cells [25]. The problem is that once these mechanisms are disrupted, disregulated kinase activation can occur, leading to disease. Targeting aberrant kinase activity with inhibitors has thus been a major

TABLE 1

Comparison of screening platforms based on direct binding

Capability	FAC–MS (Protana)	NMR	ALIS (Neogenesis)	CE-Assay™ (Cetek)	SPR (Biacore)
Throughput	High (Q1 scan); Moderate (indicator)	Moderate	High	High	Low
Protein immobilization	Yes	No	No	No	Yes
Rank compounds	Yes	Yes	No	Yes	Yes
Determine K_d	Yes	No	Yes	No	Yes
Usable chemical library	Unlimited	Yes	Limited (requires mass encoding)	Limited (parameters must be optimized)	Unlimited
Direct readout (real-time)	Yes	No	No (chromatographic separation required before MS detection)	Yes	Yes
Other potential screening applications					
Inactive proteins	Yes	Yes	Yes	Yes	Yes
Distinguish multiple binding sites	Yes	Yes	No	No	No
Protein–protein interactions	Yes	Yes	No	No	Yes
Multiple targets in single assay	Yes	Yes	No	No	No

therapeutic strategy. However, it has been established that, even in well-regulated systems, there is equilibrium between active and inactive kinases [26–28].

A major question, however, is how do you find inhibitors that favour one state over the other and take advantage of these differences when current screening methods that rely on a kinase activity readout require active kinases? Crystal structures clearly provide valuable information and insight with respect to binding but cannot be used effectively in a screening exercise. Here again the use of FAC–MS as a unique assay tool is exemplified by screening an inactive form of the EphB2 receptor kinase [29].

The Eph receptor family and several other receptor tyrosine kinases have invariant tyrosine residues in their juxtamembrane regions [30]. Autophosphorylation of these tyrosines provides a docking site for many downstream signalling proteins, but also has a crucial autoinhibitory function and has been shown to stimulate kinase catalytic activity directly [31]. It has been shown with EphB2 that mutation of the two juxtamembrane tyrosines 604 and 610 to phenylalanines [32] (Y604/610F) generated an inactive kinase. It is believed that the inability of these residues to be phosphorylated results in this EphB2 mutant remaining in a pseudo autoinhibited state.

To demonstrate the capability of FAC–MS to identify binders of an inactive kinase, both the active and inactive forms of EphB2 were evaluated under similar conditions. A mixture of four known kinase inhibitors was passed over active EphB2. Each compound exhibited because since their breakthrough times were greater than that of the void marker. The same four kinase inhibitors were screened against the inactive (Y604/610F) EphB2 kinase, and gave essentially the same results and rank order obtained in the previous experiment. It should be expected that the conformations of the ATP-binding pockets of the active/inactive kinases are similar and it is the (Y604/610F) juxtamembrane mutations that render the kinase functionally inactive. In this way, the FAC–MS results for active EphB2 act as a reference for inactive EphB2 and provide confidence that the observed binding for the inactive kinase is real. Although the structure of this inactive EphB2 has been solved, until now it would have been impossible to use this variant of EphB2 directly in a traditional functional assay.

Alternative direct binding methods

In contrast with functional assays, which require radioactive, luminescent or antibody-based methodologies, FAC–MS is a binding assay. There are other technologies currently in use that are also based on measuring direct binding, such as the surface plasmon resonance (SPR) technique from Biacore, a variety of NMR screening techniques [33–36], capillary electrophoresis (CE Assay™) from Cetek and the automated ligand identification system (ALIS) from NeoGenesis. All of these systems, including FAC–MS, have their advantages and disadvantages (Table 1). The Biacore system also requires immobilization of the protein target and, although not high throughput in nature, has the advantage that it can determine detailed kinetic analysis of binding (e.g. k_{on} and k_{off} rates) and can hence rank ligands. Recently, the coupling of MS to SPR [37] shows that it is possible to link protein detection, capture and kinetic analysis with the measurement of protein and peptide masses as well as protein identification. However, the analysis of analyte interactions with fast off-rates remains a limitation of SPR–MS. NMR-based screening approaches are becoming increasingly popular in lead discovery because of the advantages over established high-throughput functional assays. Among these, identifying very weak binders (mM K_d values) and yielding detailed structural data that indicate binding site information are most interesting. Disadvantages associated with NMR-based screening include the requirement for large quantities of protein for a typical screen, long acquisition times, isotope enrichment of

either the protein or library and complex spectra that might be difficult to analyze [35]. ALIS is similar to a capture-and-release technique and is related to FAC–MS in that MS identifies any ligands that are captured by the target but does not require protein immobilization. It is also high throughput in nature but does not generate binding data or rank ligands. It has, however, recently been shown that affinity selection-MS (AS-MS), which is related to ALIS, enables ligand ranking and classification of ligands by allosteric or direct binding site competition [38]. The high-resolution capillary electrophoresis CE Assay™ is high throughput and can rank ligands, however, it does require fluorescence labeling of the ligand or target to get data at low concentrations (low μM). Depending on its mode of use, FAC–MS can be high throughput (Q1 scan) and rank ligands or be moderate in throughput (indicator mode), and generate dissociation constants in addition to ranking ligands. The disadvantages of FAC–MS are that the protein must be immobilized in an active form and if used in a competitive fashion, an indicator with some affinity for the protein must be available. With the exception of ALIS, all methods provide real-time readout of direct binding. For any of these methods, the nonspecific binding interactions can affect the results. With FAC–MS, the nonspecific binding interactions (for example, binding to the beads, capillaries or neutral proteins, such as BSA) measured in separate experiments are always taken into account – as with the other methods they are an integral part of the screen.

Conclusion and outlook

To date, FAC–MS has been able to accommodate a wide variety of target proteins and compound libraries (including natural products), hence its applications could have a broad role in drug discovery. FAC–MS can be used as a reasonably high throughput assay tool for profiling and ranking compound mixtures. Alternatively, with the use of an indicator, only those mixtures that generate a significant % shift of the indicator need to be deconvoluted. The data to date also demonstrate that the % shift of the indicator correlates well with two significant measurements in drug discovery – IC_{50} and K_d values – and this correlation should also translate to many other proteins. FAC–MS is also well suited to tackle some of the more involved aspects of drug discovery and development, such as specificity/selectivity and evaluating protein–protein interactions.

Specificity and selectivity

The case described previously for PKCα illustrates evaluating two binding sites (i.e. the ATP- and substrate-binding sites) on the same protein. With FAC–MS, however, there is no reason why one could not use two (or more) completely different proteins to examine specificity, or two (or more) target isoforms to examine selectivity using similar protocols. As long as indicators had been identified for both proteins, monitoring indicator shifts would be straightforward. In addition, the indicators do not need to be completely independent with respect to their binding sites; this can be accounted for in their respective shift evaluations when evaluated independently.

Protein–protein interactions

One of the benefits of using MS as a detection method is its ability to not only monitor small molecules but also to monitor peptides and proteins because of the multiple charges that arise. This makes FAC–MS particularly appealing because now peptides and proteins can be used as indicators and protein–protein interactions can be evaluated directly. Moreover, FAC–MS could be applied downstream of this process to monitor proteins involved in signal transduction. Once an interesting protein has been immobilized, it could be used to probe interactions with a suspected protein – an approach that is currently performed experimentally mainly by co-immunoprecipitation [39]. It should also be noted that with FAC–MS the reverse case is possible, whereby small molecules can be immobilized and used for protein-target-fishing linking drug screening with chemiproteomics.

Immobilization strategies

FAC–MS uses similar protein immobilization strategies to those commonly found with other technologies, the major difference being that the final buffer must be FAC–MS friendly and capable of retaining protein stability. Using controlled pore glass beads (CPG), reasonably high immobilization yields were achieved and the different functionality of these beads enables a variety of immobilization methods to be used:

- Biotinylation of the protein using an activated biotin ester, followed by coupling to streptavidin- (or avidin-) coated beads through the streptavidin–biotin complex.
- Biotinylated anti-tagged monoclonal antibodies (e.g. anti-His, anti-FLAG) can be complexed with streptavidin- (or avidin-) coated beads followed by incubation with a tagged protein. With both of these methods, any remaining streptavadin (or avidin) binding sites are blocked by saturation with biotin.
- Alternatively, the protein can be attached covalently via its N-terminus to carboxylated beads. Although it is not known at present which method will be the best for a protein, the variety of proteins successively immobilized in FAC–MS studies to date is encouraging.
- Another method that is also showing promise is the incorporation of proteins with Sol-Gel (where immobilization to beads is not required) [40,41]. In recent years, it has been shown that a mild and biocompatible Sol-Gel processing method can be used to entrap active proteins within a porous, inorganic silicate matrix [42]. Dihydrofolate reductase (DHFR) Sol-Gel columns were shown to be suitable for FAC–MS screening and were used to identify known nanomolar inhibitors [40]. Sol-Gel

techniques could be particularly useful with difficult proteins, such as membrane bound GPCRs, that are typically difficult to immobilize.

The outlook for FAC–MS looks promising in both the hit identification and lead optimization processes. After hit identification, further FAC–MS screening of structurally similar compounds can be conducted to generate initial SARs. For lead optimization, combinatorial or medicinal chemistry, library expansion of the most promising hits followed by FAC–MS screening can drive the optimization. In a similar manner, FAC–MS could also be applied as a secondary screen to compounds selected using cell-based assays. There is an increasing trend in the drug discovery industry to first use cell-based assays as a primary screen to discover small-molecule hits specific to a disease condition. However, it is often difficult to discern exactly which hits are specific to the protein or pathway of interest and which are simply acting in a generic manner in the cells. Protein targets relevant to the pathway could be immobilized in the FAC–MS system and the hits from the cell-based assay screened against these targets. Those hits that interact with the target directly will be identified quickly and can be developed with the confidence that they are relevant to the disease state of interest. With all of these scenarios, FAC–MS offers very informative screening with the ability to rank ligands, distinguish binding sites, evaluate selectivity and generate binding contents.

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References

1 Geoghegan, K. and Kelly, M.A. Biomedical applications of mass spectroscopy in pharmaceutical drug discovery. *Mass Spectrom. Rev.* (in press)
2 Siegel, M.M. (2002) Early discovery drug screening using mass spectrometry. *Curr. Top. Med. Chem.* 2, 13–33
3 Min, D-H. *et al.* (2004) Chemical screening by mass spectrometry to identify inhibitors of anthrax lethal factor. *Nat. Biotechnol.* 22, 717–723
4 Boguslavsky, J. (2004) HTS assay development: Is smaller really better? *Drug Discov. Dev.* 7, 37–40
5 Gribbon, P. *et al.* (2003) Fluorescence readouts in HTS: no gain without pain? *Drug Discov. Today* 8, 1035–1043
6 Hill, D.C. (2003) Trends in development of high-throughput screening technologies for rapid discovery of novel drugs. *Curr. Opin. Drug Discov. Dev.* 1, 92–97
7 Schriemer, D.C. *et al.* (1998) Micro-scale frontal affinity chromatography with mass spectrometric detection: a new method for the screening of compound libraries. *Angew. Chem. Int. Ed. Engl.* 37, 3383–3387
8 Hage, D.S. (1997) Chromatographic approaches to immunoassays. *J. Clin. Ligand Assay* 20, 293–301
9 Hage, D.S. and Tweed, S.A. (1997) Recent advances in chromatographic and electrophoretic methods for the study of drug-protein interactions. *J. Chromatogr. B Biomed. Sci. Appl.* 699, 499–525
10 Schreimer, D.C. and Hindsgaul, O. (1998) Deconvolution approaches in screening compound mixtures. *Comb. Chem. High Throughput Screen.* 1, 155–170
11 Zhang, Y. *et al.* (1998) Immobilized nicotinic receptor stationary phase for on-line liquid chromatographic determination of drug-receptor affinities. *Anal. Biochem.* 264, 22–25
12 Kasai, K.I. and Oda, Y. (1986) Frontal affinity chromatography theory for its application to studies on specific interactions of biomolecules. *J. Chromatogr. B Biomed. Sci. Appl.* 376, 33–47
13 Zhu, L. *et al.* (2003) Frontal affinity chromatography combined on-line with mass spectrometry: a tool for the binding study of different epidermal growth factor receptor inhibitors. *Anal. Chem.* 75, 6388–6393
14 Wang, J. *et al.* (2003) Frontal affinity chromatography coupled to mass spectrometry: An effective method for K_d determination and screening of α-gal derivatives binding to anti-gal antibodies (IgG). *J. Carbohydr. Chem.* 22, 347–376
15 Luo, H. *et al.* (2003) Frontal immunoaffinity chromatography with mass spectrometric detection: A method for finding active compounds from traditional Chinese herbs. *Anal. Chem.* 75, 3994–3998
16 Chan, N.W.C. *et al.* (2002) Frontal affinity chromatography for the screening of mixtures. *Comb. Chem. High Throughput Screen.* 5, 395–406
17 Chan, N.W.C. *et al.* (2003) Frontal affinity chromatography-mass spectrometry assay technology for multiple stages of drug discovery: applications of a chromatographic biosensor. *Anal. Biochem.* 319, 1–12
18 Zhang, B. *et al.* (2001) Rapid determination of the binding affinity and specificity of the mushroom Polyporus squamosus lectin using frontal affinity chromatography coupled to electrospray mass spectrometry. *Glycobiology* 11, 141–147
19 Rich, R.L. *et al.* (2002) Kinetic analysis of estrogen receptor/ligand interactions. *Proc. Natl. Acad. Sci. U. S. A.* 99, 8562–8567
20 Slon-Usakiewicz, J.J. *et al.* (2004) Frontal affinity chromatography with MS detection (FAC-MS) of EphB2 Tyrosine Kinase Receptor. 1. Comparison with conventional ELISA. *J. Med. Chem.* 47, 5094–5100
21 Shah, A. (2004) How to choose an *in vitro* kinase assay in *Drug Discov. Dev.* March, 59-62
22 Marsilje, T.H. *et al.* (2000) The design, synthesis and activity of non-ATP competitive inhibitors of $pp60^{c\text{-}src}$ tyrosine kinase. Part 1: hydroxynaphthalene derivatives. *Bioorg. Med. Chem. Lett.* 10, 477–481
23 Slon-Usakiewicz, J.J. *et al.* (2004) Hybrid approach incorporating *in silico* and frontal affinity chromatography with MS detection (FAC-MS) screening of EphB2 tyrosine kinase receptor. *SBS 10th Anniversary Conference and Exhibition,* 11–16 September 2004, Orlando FL, USA. p.1011
24 Blume-Jensen, P. and Hunter, T. (2001) Oncogenic kinase signalling. *Nature* 411, 355–365
25 Schlessinger, J. (2003) Signal transduction. Autoinhibition control. *Science* 300, 750–752
26 Toledo, L.M. *et al.* (1999) The structure-based design of ATP-site directed protein kinase inhibitors. *Curr. Med. Chem.* 6, 775–805
27 Nagar, B. *et al.* (2002) Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). *Cancer Res.* 62, 4236–4243
28 Noble, M.E.M. *et al.* (2004) Protein kinase inhibitors: insights into drug design from structure. *Science* 303, 1800–1805
29 Slon-Usakiewicz, J.J. *et al.* Global kinase screening – new applications of Frontal Affinity Chromatography coupled to Mass Spectrometry (FAC-MS) in drug screening. *Anal. Chem.* (in press)
30 Hubbard, S.R. (2004) Juxtamembrane autoinhibition in receptor tyrosine kinases. *Nat. Rev. Mol. Cell Biol.* 5, 464–471
31 Ferguson, K.M. *et al.* (2003) EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. *Mol. Cell* 11, 507–517
32 Wybenga-Groot, L.E. *et al.* (2001) Structural basis for autoinhibition of the EphB2 receptor tyrosine kinase by the unphosphorylated juxtamembrane region. *Cell* 106, 745–757
33 Roberts, G.C.K. (2000) Applications of NMR in drug discovery. *Drug Discov. Today* 5, 230–240
34 Pochapsky, S.S. *et al.* (2001) Nuclear magnetic resonance as a tool in drug discovery, metabolism and disposition. *Curr. Top. Med. Chem.* 1, 427–441
35 Powers, R. (2002) Application of NMR to structure-based drug design in structural genomics. *J. Struct. Funct. Genomics* 2, 113–123
36 Stockman, B.J. *et al.* (2002) NMR screening techniques in drug discovery and drug design. *Progr. NMR Spectrosc.* 41, 187–231
37 Mattei, B. *et al.* (2004) Biomolecular interaction analysis and MS. *Anal. Chem.* 76, 19A–25A
38 Annis, D.A. *et al.* A general technique to rank protein-ligand binding affinities and determine allosteric versus direct binding site competition in compound mixtures. *J. Am. Chem. Soc.* 126, 15495–15503
39 Lai, K-O. *et al.* (2004) Identification of the Jak/Stat proteins as novel downstream targets of EphA4 signalling in muscle. *J. Biol. Chem.* 279, 13383–13392
40 Hodgson, R.J. *et al.* (2004) Protein-doped monolithic silica columns for capillary liquid chromatography prepared by the sol-gel method: Applications to frontal affinity chromatography. *Anal. Chem.* 76, 2780–2790
41 Cruz-Aguado, J.A. *et al.* (2004) Entrapment of Src protein tyrosine kinase in sugar-modified silica. *Anal. Chem.* 76, 4182–4188
42 Gill, I. (2001) Biol.-doped nanocomposite polymers: Sol-Gel bioencapsulates. *Chem. Mater.* 13, 3404–3421