Automated In-Line Gel Filtration for Native State Mass Spectrometry

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Characterization of protein-ligand complexes by nondenaturing mass spectrometry provides direct evidence of drug-like molecules binding with potential therapeutic targets. Typically, protein-ligand complexes to be analyzed contain buffer salts, detergents, and other additives to enhance protein solubility, all of which make the sample unable to be analyzed directly by electrospray ionization mass spectrometry. This work describes an in-line gel-filtration method that has been automated and optimized. Automation was achieved using commercial HPLC equipment. Gel column parameters that were optimized include: column dimensions, flow rate, packing material type, particle size, and molecular weight cut-off. Under optimal conditions, desalted protein ions are detected 4 min after injection and the analysis is completed in 20 min. The gel column retains good performance even after >200 injections. A demonstration for using the in-line gel-filtration system is shown for monitoring the exchange of fatty acids from the pocket of a nuclear hormone receptor, peroxisome proliferator activator-δ (PPARδ) with a tool compound. Additional utilities of in-line gel-filtration mass spectrometry system will also be discussed. (J Am Soc Mass Spectrom 2008, 19, 239–245)

Mass spectrometry (MS) is a powerful technique for the study of biomolecular complexes [1–4]. With the use of spray ionization techniques, volatile buffers at or near physiological pH, and modified time-of-flight (TOF) mass analyzers [5–7], many noncovalently bound complexes with molecular weights >0.5 MDa have been analyzed [8, 9]. A wide variety of biological interactions have been interrogated using native state mass spectrometry. For example, homo- and hetero-protein multimers, proteins associated with DNA or RNA, and proteins with peptide cofactors and small molecule ligands have all been successfully analyzed [1].

In small molecule drug discovery, an important interaction to be probed is binding of a target protein with a small molecule ligand. Native state (nondenaturing) mass spectrometry can be used to determine the presence or absence of drug ligands and in some instances, used for affinity screening [10, 11]. In the post-genomic era, nondenaturing mass spectrometry has been utilized to identify native ligands, endogenous ligands that activate or deactivate novel drug targets, for example orphan nuclear hormone receptors [12–18]. Typically, native state MS is used to detect the presence of the native ligand while additional MS experiments (GC/MS, exact mass LC/MS, etc.) provide structural elucidation of the ligand. Native ligand identification provides insight into the possible biological function of the protein and further impetus towards designing highly selective and potent drug molecules using a structure-based approach.

An additional question posed after native ligand identification is whether or not the activity of the receptor is mediated through ligand binding. If the ligand can be displaced and exchanged with a different compound, then the protein may be a bona fide drug target. However, finding the appropriate ligand exchange conditions can require screening many different ligands and solvent conditions. Native state mass spectrometry is an ideal method for ligand exchange monitoring, especially when sample preparation time is minimized.

Direct analysis of protein-ligand complexes is possible using electrosonic spray ionization [19] and nanospray [20], which are somewhat more tolerant to higher buffer salt concentrations. Usually the protein-ligand exchange mixtures contain a wide variety of other "contaminants" such as non-alkali buffer salts (HEPES and Tris), detergents (Triton, CHAPS, Brij), reducing agents (DTT), other solubilizing reagents (glycerol), and excess exchange ligand. All of these buffer components make direct detection of the protein-ligand complexes challenging.

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1044-0305/08/$32.00
doi:10.1016/j.jasms.2007.05.008

Published online May 23, 2007
Received April 4, 2007
Revised May 11, 2007
Accepted May 16, 2007

Most desalting and buffer exchange methods are off-line techniques. Some examples include molecular weight cut-off membranes or gel-filtration "spin columns." Molecular weight cut-off membranes typically require many passes to remove buffer materials and can take hours before samples are prepared. "Spin columns" are a reasonably fast and efficient way to perform buffer exchange (<20 min per sample). Both of these techniques can be multiplexed to improve throughput, but the complex must remain stable in the volatile buffers for extended periods of time before MS analysis. This is particularly important if large sample batches are to be analyzed.

A more desirable approach for improved throughput is in-line buffer exchange. Some viable approaches have been demonstrated recently, including two different on-line microdialysis devices using a fiber [21] and a microchip with laminar flow [22]. Both methods have been utilized to reduce the concentration of nonvolatile salts with a concomitant improvement in sensitivity. However, these devices were investigated with simple salt buffers and not the more complex buffer systems commonly used for solubilizing recombinant proteins.

A second approach was demonstrated by Cavanagh et al. in which they used an in-line gel-filtration column directly coupled to a mass spectrometer [23]. This paper clearly demonstrated that the technique worked for protein-protein complexes and DNA-protein complexes that were stored in complex buffer solutions and showed an attractive solution to our problems for monitoring ligand exchanges.

This work describes further advances in the use and utility of the in-line gel-filtration methodology. To improve ease of use, throughput, and robustness, the method has been fully automated using standard HPLC equipment. Also, a careful evaluation of gel packing material and the column dimensions has been conducted. Using the in-line gel column coupled to a quadrupole time-of-flight mass spectrometer, results for an unattended assay (190 injections, 2.6 days of analysis time) are shown. An example of monitoring a ligand exchange will be shown using peroxisome proliferator activated receptor delta, PPARδ, and a tool compound, a molecule which has demonstrated binding in in vitro assays and is suitable for initial in vivo studies. Additional operating modes with the in-line gel-filtration column will also be discussed.

Experimental

Materials

The in-line gel column consists of Sephadex G-25 Superfine (GE Healthcare) or Bio-Gel P6-DG fine particle polyacrylamide gel (BioRad, Hercules, CA) slurry packed, using a syringe, into a 50 mm by 1.6 mm i.d. PEEK tube (Upchurch, Oak Harbor, WA). Adapter fittings (10-32 to 1/4-28 in.) with 1/8 in. flangeless nuts and ferrule are used to connect the column to standard 1/16 in. HPLC fittings (Upchurch, Oak Harbor, WA). A 2 μm stainless steel frit is used on the 10-32 side of the adapter fitting to hold the packing material in place. Ammonium acetate (99.999%), myoglobin, ammonium hydroxide (PPB/PTFE grade), and Tris(hydroxymethyl) aminomethane hydrochloride (Tris HCl) were purchased from Sigma-Aldrich (St. Louis, MO), while glacial acetic acid was purchased from EM Science (Gibbstown, NJ). Additional recombinant proteins were obtained from collaborators at GlaxoSmithKline or University of California at San Francisco and injected as delivered.

Apparatus and Procedures

An Agilent (Santa Clara, CA) 1100 Capillary HPLC stack (thermostatted autosampler, column compartment, degasser, capillary binary pump, and an isocratic pump) was used to automate sample introduction. The binary capillary pump flowed 20 mM ammonium acetate buffer at a rate of 10 μL/min through the autosampler and into the gel column. The buffer pH was adjusted by changing the proportion of mobile phase delivered from Solvent A: pH 6 buffer or Solvent B: pH 8 buffer. The outlet of the gel column was connected to the column switching valve of the LC stack to divert lower molecular weight materials away from the ionization source. When the valve was switched, the flow rate was increased to 20 μL/min to increase the efficiency of removal of the low molecular weight substances from the column and reduce column re-equilibration time. For some experiments, the valve was not switched to monitor the elution of the salt peak. An auxiliary single channel LC pump was connected to the switching valve to maintain buffer flow through the ionization source. A typical run was completed in 20 min.

All mass spectra were collected using a modified QSTAR-XL (Applied Biosystems/MDS SCIEX, Concord, ON, Canada) quadruple time-of-flight mass spectrometer. A cylindrical sleeve was installed around the Q0 focusing quadrupole to locally increase pressure to enhance detection of intact protein-ligand complexes [6]. The instrument was set to acquire data from *m/z* 1500 to 5000 in 3s. Ions were introduced using positive ion electrospray with the orifice (DP), ring (FP), QO, and Q2 potentials optimized for each protein. The instrument was equipped with a TurboIonSpray source, which was held at a potential of +4.2 kV with the curtain gas at 20 (arbitrary units) and nebulizer at 50 (arbitrary units). No heat was applied to the nebulizer.

Results and Discussion

Characterization of the In-Line Gel Column

Recombinant protein solutions that are used to generate crystals for X-ray crystallographic studies typically are at high concentrations (>1 mg/mL) and are at or near physiological pH. To maintain solubility, many differ-

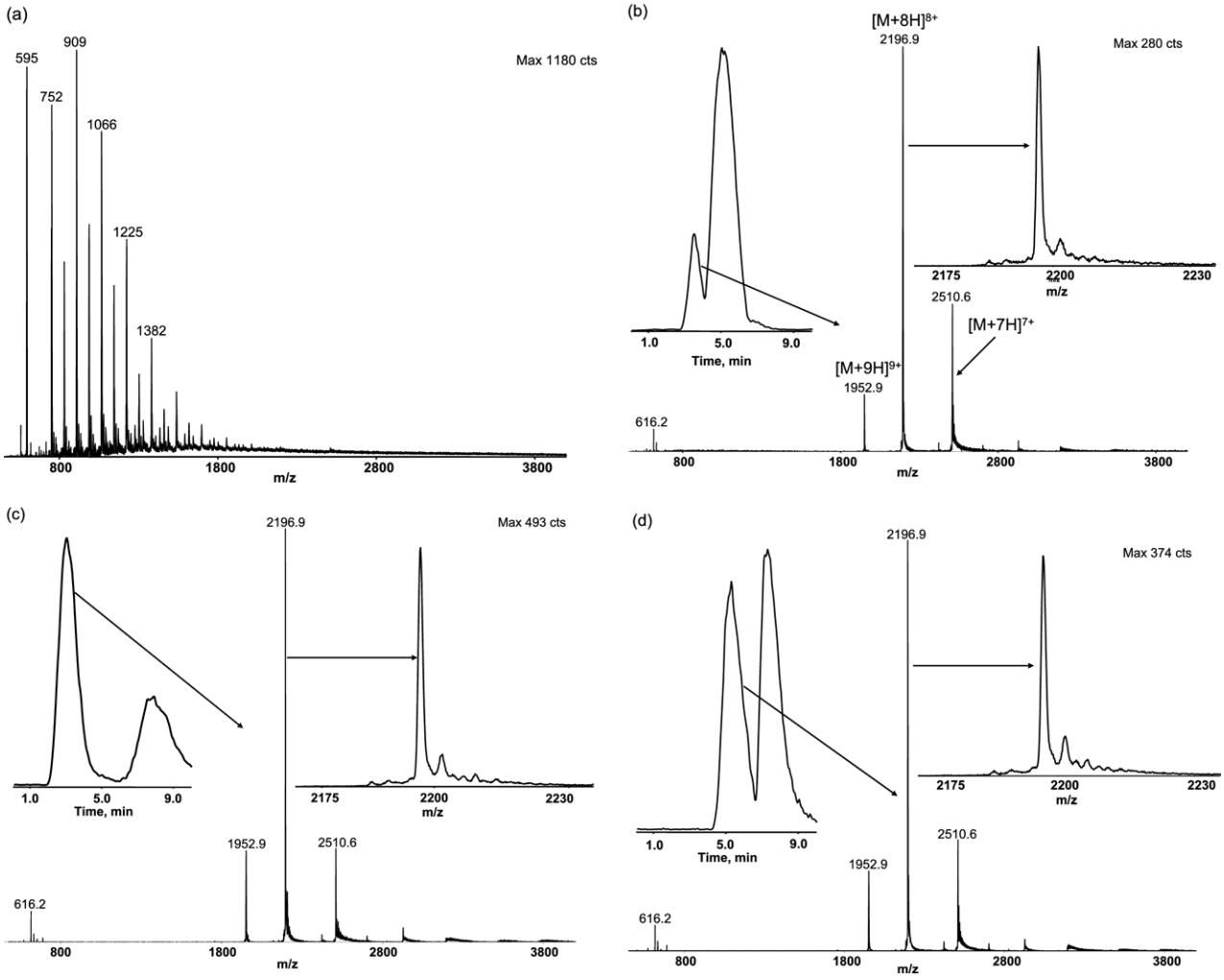


Figure 1. Mass spectra obtained for 0.5 ng myoglobin solvated in 10 mM Tris HCl buffer when analyzed under nondenaturing conditions and (**a**) without the in-line gel-filtration column, (**b**) with a 0.75 mm i.d. gel-filtration column packed with P6 gel, (**c**) with a 1.6 mm i.d. in-line gel-filtration column packed with P6 gel, and (**d**) with a 1.6 mm i.d. gel-filtration column packed with Sephedex G25. The panels on the left-hand side of (**b**)–(**d**) represent the base peak chromatograms obtained in these experiments, while those on the right-hand side display an expanded view of the $[M + 8H]^{8+}$ ion for myoglobin.

ent buffer salts, detergents, and other additives such as glycerol and DTT are used to maintain solubility and to prevent protein aggregation. Figure 1a demonstrates what occurs when myoglobin (0.1 mg/mL) is analyzed by loop injection from a relatively simple buffer, 10 mM Tris into the electrospray ionization source. Cluster ions, comprised of the buffer components, are detected while protein ions are absent, even though 500 ng of protein was loaded. Even from this low concentration buffer, clearly buffer salts and other additives adversely affect protein detection by ESI-MS.

While off-line exchange techniques such as spin-columns or molecular weight cut-off filters could be used to exchange the Tris buffer with a volatile buffer before analysis, we have observed poor recoveries (<10%) with proteins we are interested in analyzing. This is likely to be attributed to either protein binding to the membrane or reduced solubility after exchange into the assay buffer 20 mM NH_4OAc. To reach our goals of reducing the time that protein samples spend in this weak buffer system and minimizing sample preparation, we decided to explore the in-line gel-filtration method.

In in-line gel-filtration experiments, protein molecules and complexes elute before buffer salts, additives, and unbound ligands. Figure 1b shows an example when analyzing the myoglobin sample with a 0.75 mm i.d. PEEK column packed with BioRad P6 gel and using 20 mM ammonium acetate as the elution buffer via the automated system. This experimental set-up closely mimics the elution conditions described in the manual procedure described by Cavanagh et al. [23]. The mass spectrum consists of three multiply protonated ions at *m/z* 1952, 2196, and 2510, which are consistent for heme-bound myoglobin.

During initial usage of the automated system, we failed to detect many proteins, including peroxisome proliferator activated receptor delta (PPARδ) and its native ligand, (data not shown), using the 0.75 mm i.d. in-line gel column. One possible reason was a lack of resolution between the protein and salt peak. As seen in the left-hand panel of Figure 1b, the myoglobin peak is not fully resolved from the "salt" peak. At higher buffer concentration, the resolution becomes worse and a result similar to that shown in Figure 1a occurs. Other potential reasons for failing to detect proteins include providing insufficient column re-equilibration time, irreversible protein binding to the column, or protein aggregation upon buffer exchange. Additional experiments were conducted to understand these effects and to improve the performance of the column.

Among the most important parameters for increasing resolution between protein and buffer salts is matching the flow rate to the i.d. of the column. As recommended by the gel material manufacturer, the linear flow rate should range between 2 and 10 cm/h [24]. Using 0.75 mm i.d. tubing, the linear flow velocity is 136 cm/h when the flow rate is set at 10 μL/min, an ideal low flow rate for the TurboIonSpray source. By increasing the tubing size to an i.d. of 1.6 mm and using a 10 μL flow rate, the linear flow rate is reduced to 29.8 cm/h. The data shown in Figure 1c were obtained using these conditions. The protein peak is fully resolved from the buffer peak and, clearly, ion suppression effects due to Tris were minimized. Although these conditions are not within the optimal conditions for the gel-filtration media, it is impractical to increase the i.d. further since increased band-spreading and analysis time result without any further signal enhancement.

The performance of the gel-column is still suboptimal when using 20 mM buffer since the gel media should be operated with buffers at concentrations >50 mM. We have observed instances when protein recovery was greatly enhanced by increasing the buffer concentration to 200 mM. In such studies, the protein was collected off-line and analyzed using nanospray, since the TurboIonSpray source performs poorly at such high salt concentrations.

Similar gel-filtration media were also tested that had either (1) different molecular weight cut-offs ranges, for example Bio-Gel P-10, or P6 gel, or (2) different pore sizes-medium and extra fine. No significant enhancements in performance were found from these investigations. Slightly worse performance was obtained using a different type of desalting gel media, Sephadex G-25, which is composed of dextran cross-linked with epichlorohydrin gel beads, (Figure 1d). The ability to change materials by simply unpacking and repacking the PEEK columns provides an additional way to optimize analysis.

Finally, we found that the column requires a sufficient amount of re-equilibration time, e.g., time to remove low molecular weight materials from the column. We experimentally determined the re-equilibration time, by monitoring the TIC produced by background ions before injection and after the buffer peak eluted. In this simple experiment, it was determined, that the cycle time should be 20 min since the background returned to its initial level ~10 min after elution of the salt peak (~RT 8 min, Figure 1c).

The in-line gel-filtration MS system enables the possibility to perform multiple analyses in an unattended fashion since the protein remains in a favorable buffer system at a controlled temperature, i.e., 4 °C, until it is analyzed. This operational mode is useful when a given target protein is dispensed into individual vials or a 96-well plate and is incubated with a series of different compounds. Since the LC, MS, and switching valve are controlled by a computer, unattended analysis is feasible, provided that the in-line gel column is able to perform well over an extended period of time.

To test stability of the system, 0.1 mg/mL myoglobin was solubilized in (1) 20 mM Tris, (2) 20 mM NH_4OAc, or (3) 20 mM Tris, 200 mM NaCl at pH 8. In three separate experiments, each solution was analyzed ~200 times. Plotted in Figure 2 is the peak area for *m/z* 2196.9 obtained from these studies. Note that for each injection, protein was detected and *m/z* 2196.9 was the base peak for each mass spectrum (see Figure 1b and c). Clearly, from these data, the method lacks reproducibility for quantitative studies, especially when concentrated sample buffer [3] was present. However, the system has sufficient robustness to provide useful qualitative data for a large number of samples.

Interestingly, it took about 25 injections for the system to stabilize when Tris (1) or NH_4OAc (2) were used as the sample buffers. Typically, before using a freshly prepared column, four or five injections of any protein, usually 0.1 mg/mL myoglobin, are required before an appreciable signal is obtained. Perhaps some protein has to interact with the size exclusion material or other active sites to maximize the recovery of the

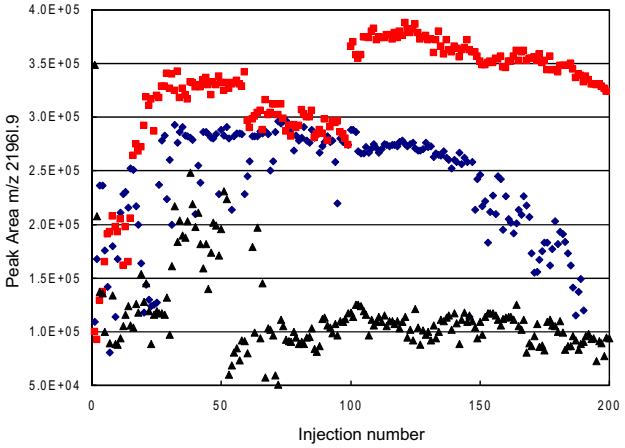


Figure 2. Integrated peak area for *m/z* 2196.9 plotted as a function of the analysis number when analyzed repetitively from 10 mM ammonium acetate (filled square), 10 mM Tris HCl (filled diamond), and 20 mM Tris, 200 mM NaCl at pH 8 (filled diamond), respectively.

protein. The signal intensity of *m/z* 2196.9 is monitored during these runs; once the counts exceed 150, the system is ready for use. For each experimental set, some extended periods of reproducible results were obtained. However, after the ~150th injection, the signal in both studies (1) and (2) decreased. Perhaps, the gel material may have broken down or may have become coated with excess protein.

With the addition of NaCl to the buffer, sodiated molecular ions became prominent, which resulted in a concomitant decrease in the signal at *m/z* 2196.9. The baseline observed before the elution of the protein peak was similar to those observed with the other buffers, which indicates that there was a sufficient amount of re-equilibration time. Possibly, a small amount of sodium is being trapped by the gel material and it only elutes when protein is present. We are currently investigating if the sodium can be exchanged from the gel material by pulsing in ammonium acetate at a higher concentration, or by the injection of a second protein to passivate the column.

At this time, it is still difficult to predict whether a particular protein will be analyzed successfully by in-line gel-filtration MS. Potential reasons for failed analyses include limited solubility of the protein in ammoniated buffers or protein requiring a particular buffer component that is removed by the column, or the buffer system itself could contain a component that fouls the column. Since the experiments can be conducted quickly, we typically seek to obtain data and then sort out what factors contribute to the analysis failing.

Use of In-Line Gel Filtration MS in Drug Discovery

The in-line gel-filtration MS technique was initially used to determine the presence of native ligands in the orphan nuclear hormone receptors: human steroidogenic factor-1 (SF-1) and liver receptor homolog 1 (LRH-1), which were over-expressed and purified from bacterial°cells°[18].°In°these°studies,°it°was°discovered that both proteins had the binding pocket occupied with diacyl phosphatidyl glycerols (PG) and ethanolamines°(PE),°Figure°3a.°The°in-line°gel°technique°enabled us to rapidly examine a series of samples that clearly demonstrated that both PG and PE ligands could be exchanged with phosphatidyl inositols (P4,5I and P3,4,5I). The PI ligands were subsequently proposed to be the native ligands found in eukaryotic cells. Recently, a sample of hSF-1 recombinantly over-expressed by *baculovirus* in Sf9 insect cells, was analyzed using native state mass spectrometry. Phosphatidyl choline (760 Da) and phosphatidyl inositol (834 Da) were identified as noncovalently bound native ligands, as°is°shown°in°Figure°3b.

The in-line gel MS technique is ideally suited for monitoring° ligand° exchange° experiments.° Figure° 4

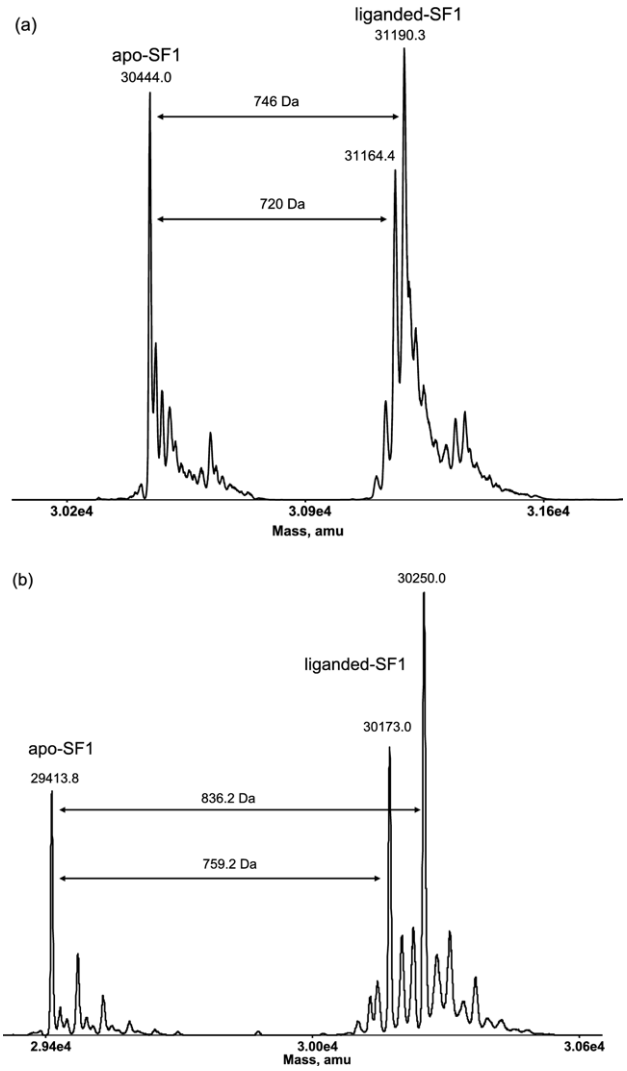


Figure 3. Deconvoluted native state mass spectra obtained from the analysis of human steroidogenic factor-1 (hSF-1) for expressed from (**a**) *E. coli* and (**b**) sf9 cells using *baculovirus*. In each spectrum the apo-protein (i.e., mass = 30,444 Da in (**a**) was detected along with multiple liganded proteins. By changing the expression system, different native ligands were observed; (**a**) phosphatidyl glycerols (Δmass = 720 and 746 Da) and ethanolamines (not labeled) and (**b**) phosphatidyl choline (Δmass = 759 Da) and phosphatidyl inositol (Δmass = 836 Da). Structure elucidation of the ligands was achieved in separate experiments using accurate mass and MS/MS experiments (data not shown).

shows an example of displacement of weakly bound (~1 μM) fatty acids (*m/z* 253 and *m/z* 283), which are native ligands for PPARδ, with a tight binding compound (EC_{50} = 1 nM, Structure **1**), which has a nominal MW°of°471.5°Da°[25].°The°extent°of°ligand°exchange°is varied by adjusting the ligand to protein ratio between: (1) no ligand added, (2) to an excess of protein 1:2, and (3) to an excess of ligand 5:1. Clearly, the ligand completely displaces the fatty acids when introduced in excess.

Displacement of the native ligand becomes more difficult when it is more tightly bound to the receptor, the exchange ligand has a weaker binding affin-

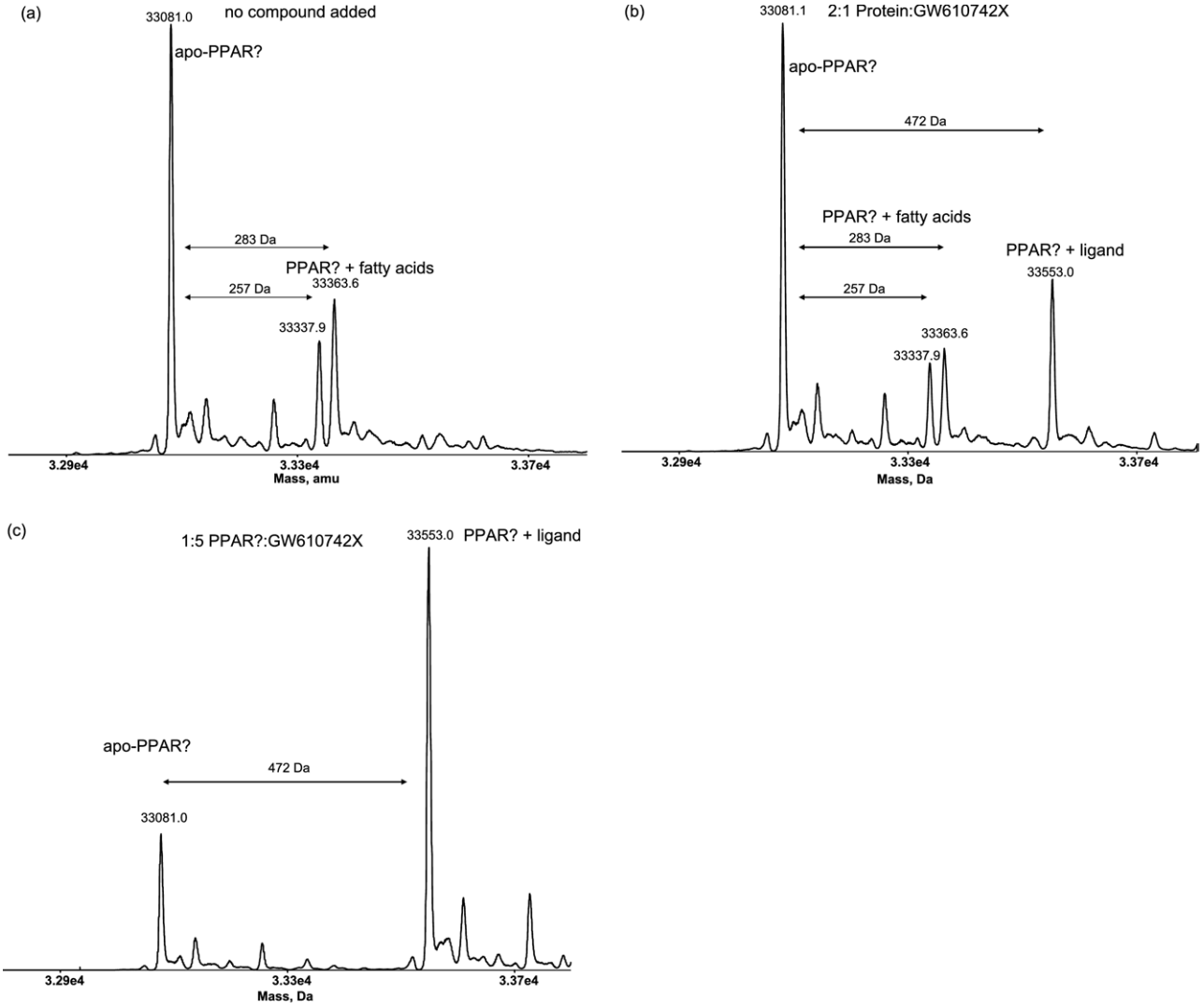
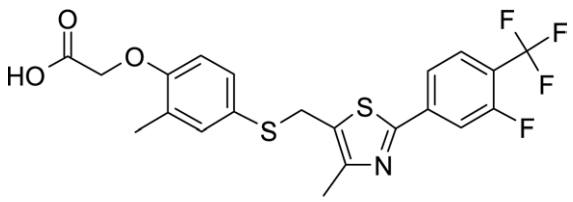


Figure 4. Experiment conducted to mimic the monitoring of endogenous ligands (fatty acids, 253 and 281 Da) with GW610742X (MW 471.5 Da). The ligand to protein ratio was varied from (**a**) 0, no ligand introduced, to (**b**) 1:2, ligand as the limiting reagent, and (**c**) 5:1, ligand in excess. Note that the samples were analyzed within 1 min of the addition of the ligand to the protein solution.

ity, or the endogenous ligand has low solubility in the exchange buffer. These are the typical samples provided for analysis to support early drug discovery efforts. In such systems, multiple in-line gel-filtration MS analyses are conducted to determine the extent of ligand exchange under a variety of different exchange conditions while using only a small amount of protein (<0.1 mg). The exchange conditions may be adjusted in one or more of the following ways: addition of reagents to unfold and then refold the protein, adjustment of temperature, pH, ligand concentration, additives to improve solubility of the endogenous ligand, and incubation time (3 h to 2 wk). If the temperature is not a variable for the exchange, samples can be incubated in vials in the autosampler and analyzed at times specified by the collaborator. Once the optimal exchange conditions are established, the remainder of the protein (100 mg or more) is ligand-exchanged, resulting in a solution designated for crystallization trials. If diffracting crystals are obtained, these crystals are analyzed by X-ray crystallography to potentially achieve a solved protein-ligand crystal structure.



GW610742X

Structure 1

Conclusions

In-line gel-filtration provides a convenient means to introduce samples for native state mass spectrometry. The use of an the in-line gel column and a modern LC system enables fully automated sample analysis and maximizes the time protein complexes are stored in favorable buffers before analysis. This method has been successfully used for the analysis of many different types of protein complexes.

There are, however, instances when the in-line gel-filtration method fails. Usually this is attributed to the protein becoming insoluble or aggregating when sample buffers are exchanged with the 20 mM ammonium acetate on the column. Typically, the column becomes plugged and a new one needs to be constructed. Sometimes, increasing the strength of the buffer to 200 mM and collecting the contents for nanospray or increasing or decreasing the pH provides an effective alternative strategy. Perhaps another possible solution to be explored is to couple the in-line gel-filtration column operated with higher strength buffers to the microdialysis device. The in-line column could thus be operated under its optimal conditions and would remove other small molecules that could foul the microdialysis chip. With further improvement in robustness, it could be conceivable to develop a protein-ligand complex screening "open-access" [26, 27] "work" stations "based" upon the in-line gel MS technique.

Acknowledgments

The authors thank Tim Willson and Shawn Williams for their continued support on orphan nuclear receptor research at GlaxoSmithKline. They also thank Holly Ingraham, Irina Krylova, and coworkers at UCSF for providing them with the *E. coli* sample of hSF-1. They also thank Igor Chernushevich for installing the IonCooler and his advice for optimizing the mass spectrometer for nondenaturing mass spectrometry.

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