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Determining Affinity-Selected Ligands and Estimating Binding Affinities by Online Size Exclusion Chromatography/Liquid Chromatography−Mass Spectrometry

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Size exclusion chromatography (SEC) isolation of affinity-selected ligands combined with reverse phase liquid chromatography−mass spectrometry (LC-MS) is an effective means for identifying members of mixtures which form tightly bound noncovalent complexes with target proteins. A potential liability of the approach is that the SEC isolation is carried out under nonequilibrium conditions favoring protein/ligand complex dissociation. At long SEC isolation times and/or for complexes with fast off-rates the extent of dissociation can jeopardize the ability to detect the affinity-selected components. Additionally, equilibrium binding affinities cannot be exactly determined from the measured distribution of isolated ligands. We present here an online SEC/LC-MS system for determining affinity-selected members of active mixtures which reduces this liability. A kinetic model of the SEC isolation process is developed to determine the practical limits for the application of the method and to extrapolate equilibrium binding affinities from the nonequilibrium data. The utility of online SEC/LC-MS for identifying affinity-selected ligands and for estimating binding affinities is demonstrated for a small molecule mixture of compounds with known binding affinities and for a simple combinatorial mixture.

Introduction

Combinatorial chemistry is utilized throughout the pharmaceutical industry as a means to rapidly amplify chemical diversity in the search for new therapeutic leads. The accelerated pace at which new chemical entities are produced by this approach has created many new challenges in characterizing compounds and screening compounds for activity. A commonly used strategy for increasing the rate at which new compounds can be screened for activity is to assay mixtures of compounds and subsequently deconvolute those mixtures which exhibit significant activity to find the active ligands. The deconvolution methods range from simple discrete resynthesis/screening of all members of the active mixture to fractionation of active mixtures followed by screening and characterization of the active fractions to a wide range of affinity selection based strategies utilizing either immobilized[1,2] or solution-phase[3−12] protein. The immobilized protein approaches have the potential liability that the affinity of the target protein may be changed by immobilization. Solution-phase affinity selection approaches take advantage of the ability of active compounds to form tightly bound noncovalent complexes with the target protein. The protein/ligand complexes are then either determined directly using electrospray ionization−Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR MS)[3,4] or separated from the unbound/inactive components based on their size, usually using ultrafiltration[5−7] or size exclusion chromatography (SEC),[8−12] followed by denaturation of the complex and detection of the active ligand by either ultraviolet spectroscopy or mass spectrometry.

An online SEC strategy for isolating affinity-selected components followed by reverse phase liquid chromatography−mass spectrometric (LC-MS) detection of the selected ligands has many of the attributes needed to support an active combinatorial leads discovery program. The approach is sensitive, rapid, rugged, and easily automated. The general validity and utility of the SEC isolation/LC-MS detection approach for determining ligands which have been affinity-selected from combinatorial mixtures has been demonstrated for a number of target proteins.[8−12] However, this strategy has an inherent liability which has not been adequately addressed in the literature. During SEC isolation the separation of the complex from the free ligand disturbs the equilibrium condition such that the reverse reaction (the dissociation of the protein/ligand complex) is favored. This creates two problems. First, if the extent of dissociation is large during the SEC isolation, due to either a large off-rate for the complex or a long SEC isolation time, the recovery of the complex will be poor and the ability to detect the affinity-selected ligand may be compromised. Second, the distribution of ligands recovered by the SEC isolation is not equal to the equilibrium distribution of protein/ligand complexes. Consequently, exact equilibrium binding affinities cannot be determined directly from the measured distribution.

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Hegy et al. have found a good correlation between Elisa measured affinities and the distribution of affinity-selected/SEC-isolated ligands for a series of cyclosporines.[12] It appears that reasonable estimates of relative equilibrium binding affinities can be obtained for complexes with half-lives that are long relative to the SEC isolation time. However, it is certain that within the diversity of chemical scaffolds of interest for pharmaceutical research there are many compounds for which this will not be the case. For these compounds the correlation between the measured distribution of affinity-selected ligands and the equilibrium binding affinities will be poor. A discussion on the nature of the issue, the magnitude of the problem, and strategies to minimize or eliminate the liabilities is clearly needed.

In this article we present an approximate model for the nonequilibrium kinetics of complex dissociation during SEC isolation. This kinetic result is combined with equations describing equilibrium complex formation to illustrate and investigate the limitations of the SEC isolation approach. Strategies for increasing SEC recovery efficiency and for extrapolating absolute and relative equilibrium binding affinities from the nonequilibrium data are proposed and demonstrated.

An online SEC/LC-MS system utilizing BioSep silica packed SEC guard (4.6 mm × 30 mm) columns for the isolation step is described. The BioSep SEC columns can withstand pressures up to 1000 psi thus permitting the use of relatively high flow rates. The high flow rates combined with the short length of the columns enable relatively rapid isolation of the complexes from the incubation mixture, thereby reducing liabilities associated with complex dissociation during the isolation step. The analysis procedure presented here was designed to validate the online SEC/LC-MS approach for determining affinity-selected members of mixtures, to investigate complex dissociation during SEC isolation, and to test approaches for estimating equilibrium binding affinities from the nonequilibrium data. The procedure is not optimized to achieve maximum throughput nor are the capabilities of the LC-MS system for identifying selected ligands fully exploited in this work. These topics will be featured in future reports.

Two small molecule libraries were used to validate the online SEC/LC-MS approach and demonstrate the kinetic models developed here: a mixture consisting of four diverse compounds with known affinities for the target protein, matrix metalloprotease (MMP3), and a small (36 member) combinatorial mixture which exhibited activity in a high-throughput MMP3 screen.

Experimental Section

The target protein was recombinant human matrix metalloprotease (MMP3) obtained as previously described.[13] The test mixture contained four proprietary compounds: two of which are MMP3 active (YZ103 with a binding affinity, K_D, of 1 nM and SN476 with a binding affinity of 33 nM) and two of which are MMP3 inactive (SN474 and SN478, both with dissociation constants much greater than 50 μM). The equilibrium dissociation constants for these compounds were determined as previously described by Copeland et al.[13] The combinatorial mixture consisted of 36 amino acids with proprietary end groups synthesized on acid cleavable resin (Wang) and dissolved to an overall concentration of 10 mM in DMSO. The matrix used for the affinity selection incubation was 100 mM Tris, 2 mM $CaCl_2$, 1 mM NaCl, at pH 7.4. Two sets of affinity selection conditions were used in these investigations: "excess protein" conditions in which the protein concentration was 4 μM and the ligands were present at 1 μM each and "protein limited" in which the target protein was present at 1 μM and the concentrations of the ligands were greater than 10 μM each. The protein/ligand mixtures were incubated at room temperature for 1 h. The online SEC/LC-MS analysis sample size was 20 μL; thus the maximum quantity of protein/ligand complex used per analysis (under either incubation conditions) was 20 pmol.

The instrumental setup and sequence of events for the online SEC/LC-MS analysis are outlined in Figure 1. The procedure can be divided into three major phases: (1) the protein/ligand complexes and unbound ligands are separated by SEC, and the complexes are collected on the protein trap; (2) the trapped material is desalted; (3) the LC gradient denatures the trapped complexes and elutes the released ligands into the LC-MS system. The detailed timeline for an analysis (at a SEC flow rate of 400 μL/m) is given in Table 1.

All liquid chromatographic components in Figure 1 are HP1100 modules. The mass spectrometer is the Micromass Q-Tof Hybrid instrument operated in the positive electrospray (ES^+) TOF-MS mode. The mass range acquired was 250−550 Da in 1 s. The SEC column is a 4.6 mm × 30 mm Phenomenex BioSep SEC-S2000 guard column. The SEC mobile phase is 10 mM potassium phosphate buffer with 2% acetonitrile at approximately pH 7.4; the trap desalting is done with 100% aqueous. The trap is a Michrom Bioresources 1 mm × 10 mm Micro Protein Trap. The LC column is a 2 mm × 50 mm Zorbax XDB-C8 LC-MS column. The gradient LC mobile phase A is 0.1% acetic acid in water and B is acetonitrile with 0.1% acetic acid.

Results

SEC Fractionation. Figure 2 shows the SEC−UV chromatograms (Hewlett-Packard DAD, 190−300 nm) obtained separately for the target protein and the four ligands in the test mixture. There is significant disparity in the retention times of the four ligands which does not correlate to the differences in the molecular weights. The differences in retention time are probably due to nonspecific interactions of the diverse structure scaffolds with the SEC packing. The MMP3 peak exhibits a significant tail, perhaps also due to nonspecific interactions of the protein with the packing. All of the ligands are at least partially resolved from the protein. The SN478 does overlap somewhat with the tail of the protein peak. The cutoff time for collection of the proteinacous fraction in the SEC/LC-MS analysis (Table 1) was selected so as to collect as much of the proteinacous fraction as possible while eliminating all unbound ligand from the

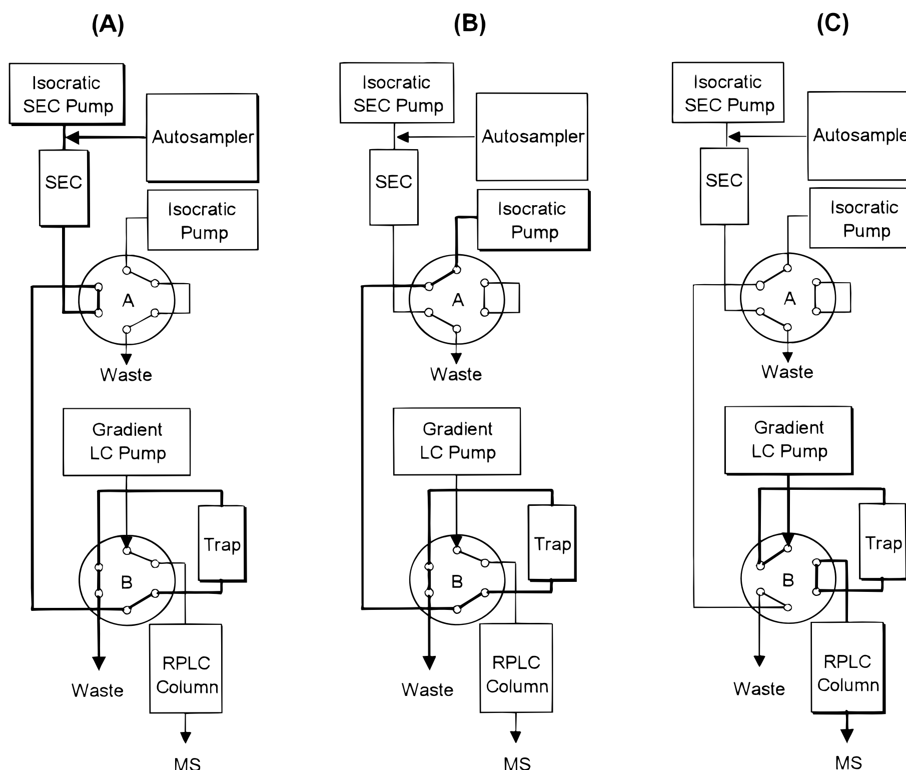

Figure 1. Instrumental setup and sequence of events for online SEC/LC-MS analysis of affinity-selected ligands: (A) inject affinity-selected mixture and trap proteinacous fraction, (B) desalt trap, (C) denature complex(es) and elute ligand(s) for LC-MS determination and remove unbound ligands from SEC column.

Table 1

reference in Figure 1	analysis time	column switch A	gradient pump B	column switch B	comment
1	0.00	pos 1	2% B @ 100 μL/min	pos 2	inject sample
	0.50			pos 1	begin trapping proteinacous fraction
2	1.25	pos 2			end trapping of proteinacous fraction, desalt trap, send unbound ligands to waste
3	6.25		2% B @ 100 μL/min	pos 2	denature/elute trapped material into LC-MS
	11.25		100% B @ 100 μL/min		
	21.25		100% B @ 100 μL/min		end LC-MS analysis
	21.35		2% B @ 100 μL/min		return to initial conditions

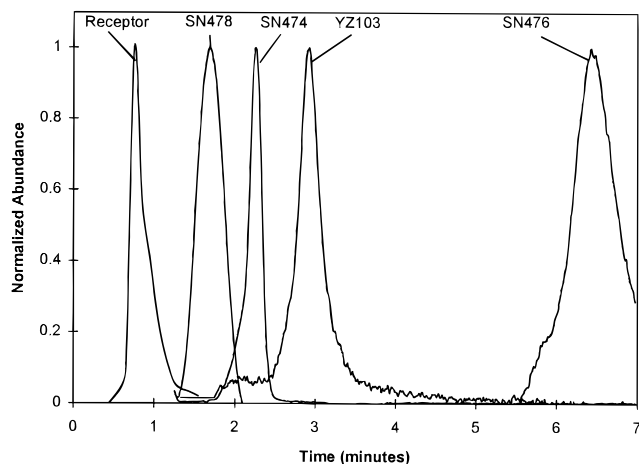

Figure 2. SEC−UV traces obtained separately for the receptor and four test compounds at a flow rate of 0.4 mL/min.

trapped fraction. We estimate that more than 95% of the proteinacous fraction is recovered under these conditions.

Determination of Affinity-Selected Ligands. The ability of the online SEC/LC-MS procedure to detect and identify affinity-selected ligands was established using the excess protein incubation of the four-component test mixture with MMP3. Under these conditions the equilibrium concentrations of the protein/ligand complexes for the two active compounds (YZ103 and SN476) are essentially equal to the initial concentrations of free compound, 1 μM each. The equilibrium complex concentrations for the two inactive compounds (SN474 and SN478) are estimated to be less than 0.01 μM each.

Two control experiments were employed to validate the affinity selection results. The negative control consists of the SEC/LC-MS analysis of the ligand mixture without protein present: all of the ligands are unbound and should be eliminated by the SEC fractionation. This control confirms that the SEC fractionation excludes all unbound ligands and validates that ligands observed in the analysis of the protein/ligand incubation are due to affinity-selected ligands. The positive control entails analyzing the protein/ligand incubation without SEC fractionation: all ligands (both bound and free) are conveyed to the protein trap for subsequent LC-MS analysis. This control validates that all components of

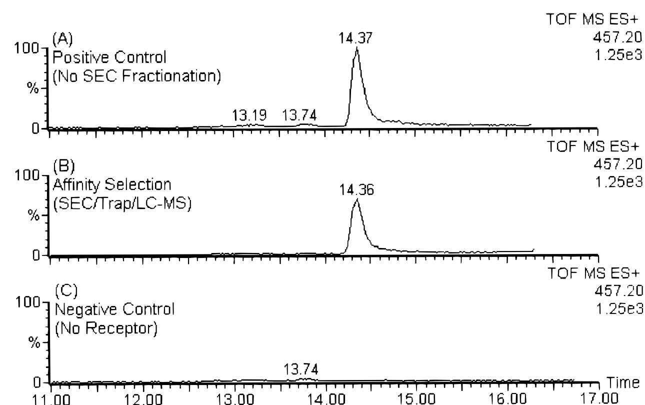

Figure 3. Selected ion traces for active compound YZ103, $[M + H]^+$ at m/z 457: (A) positive control, (B) affinity-selected sample, (C) negative control.

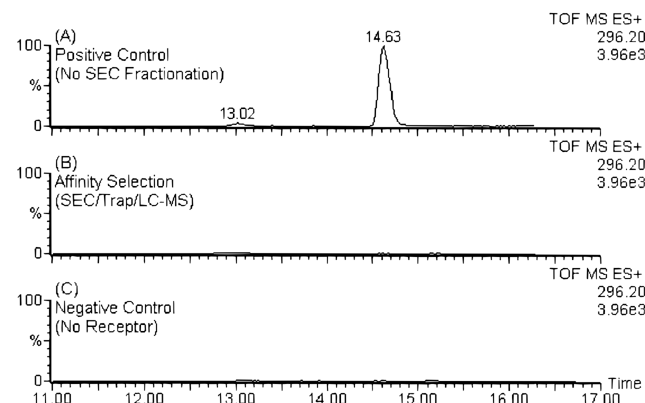

Figure 4. Selected ion traces for inactive compound SN478, $[M + H]^+$ at m/z 296: (A) positive control, (B) affinity-selected sample, (C) negative control.

the mixture can be trapped and detected by LC-MS and will serve as a calibration for determining binding affinities.

Figure 3 shows the selected ion traces for the $[M + H]^+$ of the active ligand YZ103, m/z 457, observed in the online SEC/LC-MS analysis of the affinity selection mixture and in the negative and positive controls. No YZ103 is observed in the negative control, Figure 3C, establishing that the SEC fractionation does eliminate all unbound YZ103 from the trapped proteinacous fraction. Therefore, any YZ103 observed in the analysis of the protein/ligand incubation must be due to complexed (affinity-selected) ligand. The YZ103 is observed in the positive control, Figure 3A. The integrated area under the selected ion trace represents the instrument response for the total quantity of YZ103 in the analyzed volume of the incubation mixture (i.e., the response for the 20 pmol of YZ103 in the 20 μL sample analyzed). Figure 3B shows the response for YZ103 in the SEC/LC-MS analysis of the affinity-selected mixture. A significant response at the correct mass and retention time for YZ103 is observed. The response is approximately 70% of that expected for the total quantity of YZ103 in the incubation mixture (the response observed in the positive control).

The selected ion traces observed for the inactive compound SN478 ($[M + H]^+$ at m/z 296) in the affinity selection analysis of the test mixture and the positive and negative controls are shown in Figure 4. The negative control, Figure 4C, confirms that SEC isolation has excluded all free SN478.

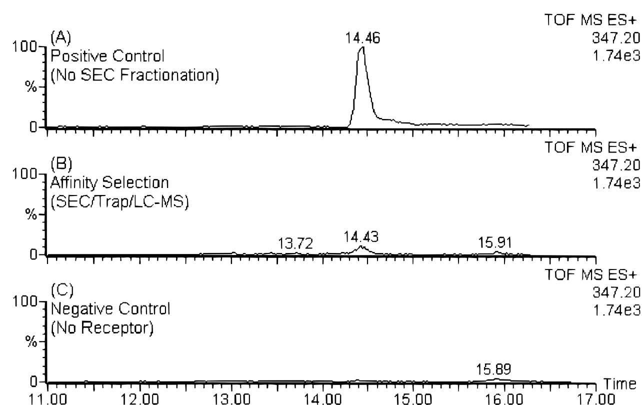

Figure 5. Selected ion traces for active compound SN476, $[M + H]^+$ at m/z 347: (A) positive control, (B) affinity-selected sample, (C) negative control.

(SN478 is the ligand least resolved from the protein by the SEC fractionation; see Figure 2.) The positive control confirms that SN478 is present in the protein/ligand mixture and that the ligand can be detected by the trap/LC-MS method, Figure 4A. No signal corresponding to SN468 is observed in the affinity selection/SEC/LC-MS experiment, Figure 4B. Similar results were obtained for the other inactive compound in the mixture, SN474.

Figure 5 shows the results for the second active ligand in the test mixture, SN476 ($[M + H]^+$ at m/z 347). Again the positive and negative controls confirm that the ligand can be detected and that the SEC isolation does remove all unbound ligand from the affinity-selected mixture, Figure 5A,C. The SN476 is detected in the affinity selection/SEC/LC-MS experiment, Figure 5B, but the recovery is less than 20% (compared to the response in the positive control). Under excess protein affinity selection conditions the equilibrium concentration of SN476 is approximately equal to that of the YZ103. The lower recovery of SN476 is due to greater dissociation of the complex during the SEC isolation step (see below).

Protein/Ligand Complex Dissociation during SEC Fractionation. Both of the active compounds (YZ103 and SN476) exhibit relatively low recovery in the online SEC/LC-MS analysis (compared to the positive control). The losses are due to dissociation of the protein/ligand complexes during the SEC fractionation. The SEC isolation was performed as rapidly as was considered prudent without risking interference from unresolved free ligands; the SEC isolation time (the retention time for the void volume which contains the complexes) was approximately 43 s. The low fractional recoveries for these two compounds indicate that losses due to dissociation can be significant and will likely be problematic for some classes of compounds. Additionally, the distribution of the recovered ligands are not equal to the expected equilibrium distribution of the corresponding complexes. Therefore, exact equilibrium binding affinities cannot be obtained directly from the measured distribution of ligands. For these reasons it is important to understand the kinetics of complex dissociation during SEC isolation and how this affects the observed distribution of affinity-selected components.

For the reversible reaction of ligand, L, with protein, R, to form the noncovalently bound complex, C

$$\mathrm{R} + \mathrm{L} \underset{K_r}{\overset{K_f}{\rightleftharpoons}} \mathrm{C} \qquad (1)$$

the equilibrium dissociation constant is

$$K_D = [\mathrm{R}][\mathrm{L}]/[\mathrm{C}] \qquad (2)$$

During SEC fractionation of the affinity-selected mixture the complex (and unbound protein) elutes in the void or exclusion volume while the unbound ligands are retained in the inclusion volume of the SEC packing and elute later. Any free ligand generated by unimolecular dissociation of the complex (the reverse reaction in eq 1) during SEC fractionation is also removed from the void volume by inclusion into the SEC packing. If the concentration of free ligand in the void volume is immediately reduced to zero, then the time-dependent concentration of protein/ligand complex during SEC fractionation is

$$\delta[\mathrm{C}]/\delta t = -K_r[\mathrm{C}]$$

Solving the rate equation for the concentration of the complex, [C], at time t gives

$$\ln([\mathrm{C}]/[\mathrm{C}]_{eq}) = -K_r t \qquad (3)$$

where $[\mathrm{C}]_{eq}$ is the initial or equilibrium concentration of the complex. If t is the retention time of the complex (which is equal to the void volume time) then [C] represents the concentration of the complex reaching the trap. Once the complex is trapped, further dissociation is irrelevant since ligand released after this point is retained by the trap. Since a ratio of molar quantities for two species in a given volume is equal to the ratio of concentrations of those species, the ratio in eq 3 may be defined as the ratio of molar quantities in the analyzed volume. In this way eq 3 remains valid even if the concentrations change due to dilution or diffusion during the SEC isolation.

Of course, unbound ligand is neither instantly nor completely removed from the SEC exclusion volume. Consequently, the ligand concentration in the exclusion volume is not absolutely zero nor is the dissociation time exactly equal to the void volume time. Nonetheless, a useful, albeit approximate expression is obtained by making these two assumptions then multiplying $[\mathrm{C}]/[\mathrm{C}]_{eq}$ in eq 3 by $[\mathrm{L}]_0/[\mathrm{L}]_0$ (where $[\mathrm{L}]_0$ is the amount of ligand initially added to the incubation mixture) and rearranging

$$\ln([\mathrm{C}]/[\mathrm{L}]_0) = -K_r t + \ln([\mathrm{C}]_{eq}/[\mathrm{L}]_0) \qquad (4)$$

Because the molar quantity of complex is measured indirectly as the quantity of ligand released upon denaturation of the complex, the mass spectrometric response factors for the "complex" and the unbound ligand are the same. Thus eq 4 can be written as

$$\ln(L/L_0) = -K_r t + \ln([\mathrm{C}]_{eq}/[\mathrm{L}]_0) \qquad (5)$$

where L and L_0 are the mass spectrometric responses for the ligand recovered from the trapped/denatured complex and for the ligand at its initial concentration (which is equivalent to the total of the free and bound ligand as determined in the positive control). Equation 5 describes a straight line with a slope equal to the negative of the off-rate for the complex and an intercept equal to the equilibrium concentration of the complex relative to the initial concentration of the ligand, $[\mathrm{C}]_{eq}/[\mathrm{L}]_0$ (which is a fixed value for a given ligand/protein pair and set of incubation conditions).

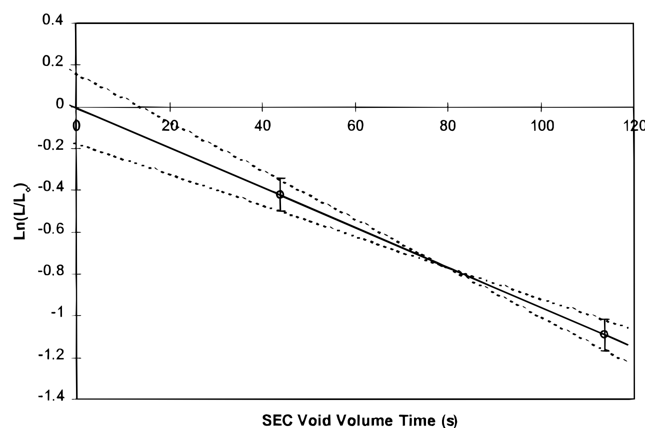

Figure 6. Estimation of absolute binding affinity for YZ103: log of the ratio of responses for the ligand in the affinity selection experiment and that in the positive control vs SEC void volume time. Data points represent the average values for three measurements; error bars represent one standard deviation.

Figure 6 illustrates the behavior described in eq 5 for the MMP3/YZ103 complex. Excess protein affinity selection conditions were used in order to eliminate complications due to competitive binding between YZ103 and SN476. The mass spectrometric response for the YZ103 released from isolated complex, L, was determined at two retention times by varying the SEC flow rate. The response for the ligand at the initial concentration, L_0, was determined by injecting the protein/ligand mixture directly onto the protein trap (the positive control experiment) and measuring the total response for the ligand. A compound closely related to the ligand was injected directly onto the trap immediately after trapping the proteinacous fraction from the SEC and prior to desalting the trap. The response from this compound served as an internal standard to compensate for fluctuations in the mass spectrometric response factor. The data points in Figure 6 represent the average values for three determinations, and the error bars represent one standard deviation. The decrease in the fractional recovery of the complex, $\ln(L/L_0)$, with increasing retention time in Figure 6 is consistent with unimolecular dissociation of the complex during SEC isolation.

The equilibrium concentration of the protein/ligand complex can be calculated from the initial incubation conditions and the known binding affinity by expressing the equilibrium concentrations of the protein and ligand in terms of the equilibrium concentration of the complex,

$$[\mathrm{L}]_{eq} = [\mathrm{L}]_0 - [\mathrm{C}]_{eq} \qquad (6a)$$

$$[\mathrm{R}]_{eq} = [\mathrm{R}]_0 - [\mathrm{C}]_{eq} \qquad (6b)$$

substituting these expressions into eq 2, and finally solving

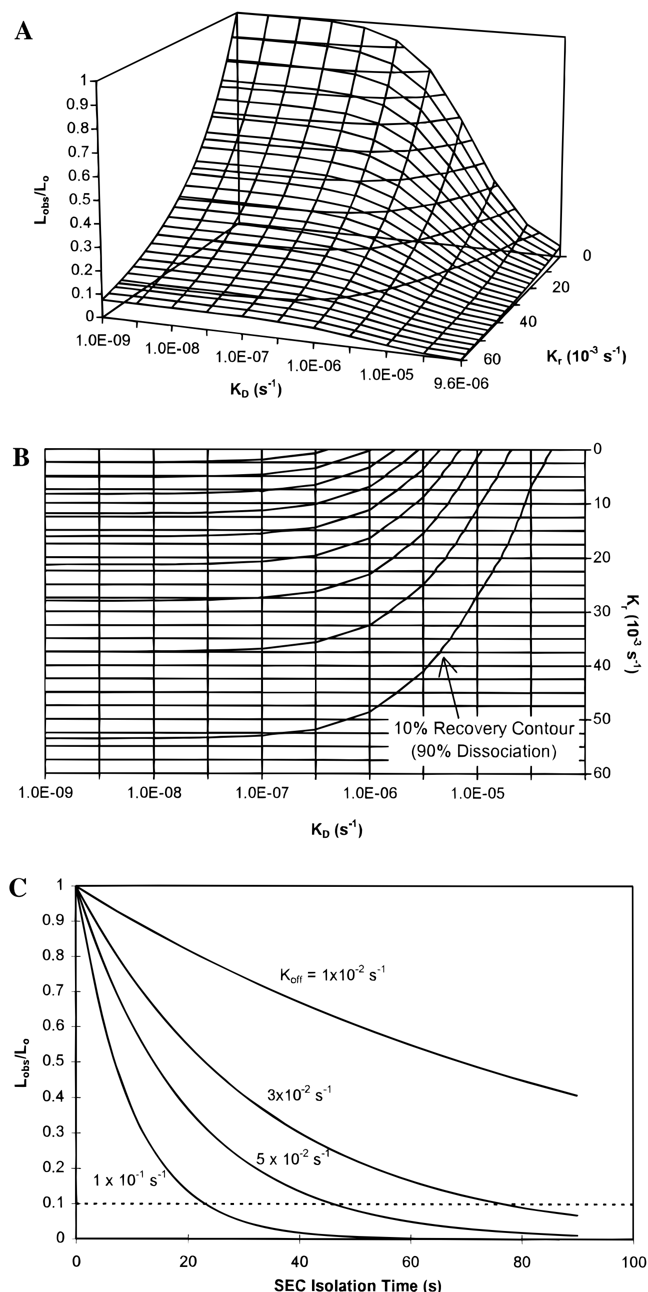

Figure 7. (A) Three-dimensional plot of the fractional amount of ligand recovered relative to the initial amount as a function of binding affinity, K_D, and off-rate, K_r. (B) Contour plot of fractional recovery. (C) Fractional recovery as a function of the SEC isolation time.

the quadratic equation:

$$[C]_{eq} = \{([R]_0 + [L]_0 + K_D) - \sqrt{([R]_0 + [L]_0 + K_D)^2 - 4[R]_0[L]_0}\}/2 \quad (7)$$

Taking the exponential of eq 5 gives an expression describing the fraction of the complex recovered by the SEC fractionation of the incubation mixture:

$$L/L_0 = [C]_{eq}\, e^{-K_r t}/[L]_0 \quad (8)$$

With eqs 7 and 8 the effects of the principal parameters influencing the affinity selection/SEC isolation procedure can be explored. Figure 7A shows the fraction of ligand recovered by SEC fractionation (relative to the initial quantity of ligand in the incubation) at varying values for the binding affinity, K_D, and the off-rate, K_r. In this example the SEC isolation time, t, is 43 s and the initial concentrations of protein and ligand are 4 and 1 μM, respectively. Under these conditions, the fractional amount of ligand recovered is nearly independent of binding affinity for values of K_D less than about 1 μM. Between about 1 and 10 μM the fractional recovery of ligand drops off precipitously. The exponential dependence on K_r produces a rapid decrease in fractional recovery with increasing K_r. For a complex with an off-rate of 3×10^{-2} s^{-1} (the estimated off-rate for SN474, see below), nearly 80% of the complex is lost to dissociation during the 43 s SEC isolation.

A contour plot of this same information, Figure 7B, is more helpful for determining the useful limits of the affinity selection/SEC/LC-MS analysis under a specific set of experimental conditions. For example, if we (somewhat arbitrarily) define the criterion for definitively establishing an active component as one for which the measured response in the SEC/LC-MS analysis is at least 10% of that in the positive control, then those ligands which will be "detected" by the affinity selection/SEC/LC-MS analysis are those with combinations of K_D and K_r encompassed within the 10% recovery contour in Figure 7B. Note that a compound with a 30 μM binding affinity will be detected if it has an off-rate of only 5×10^{-3} s^{-1} ($t_{1/2} = 138$ s) while a 1 nM compound will not be detected if it has a large off-rate of 6×10^{-2} s^{-1} ($t_{1/2} = 11.5$ s). Clearly, it will be important to be aware of these relationships when applying this method and interpreting the data.

These relationships can also be used to help develop conditions appropriate for the known characteristics of the target protein and ligands being screened. Figure 7C shows the time-dependent dissociation profiles calculated with eq 8 for ligands with 1 nM binding affinities and differing off-rates. Again, with the detection criterion set as 10% recovery (relative to the positive control) we find that the ligand with an off-rate of 1×10^{-2} s^{-1} ($t_{1/2} = 69$ s) is easily detectable even with SEC isolation times of more than 3 min. On the other hand, to detect the ligand with an off-rate of 10×10^{-2} s^{-1} ($t_{1/2} = 7$ s) requires an SEC isolation time of less than 25 s.

Estimating Absolute K_D Values and Off-Rates. The slope and intercept of the line exhibited in Figure 6 are -9.6×10^{-3} s^{-1} and -0.00317, respectively. From eq 5, the off-rate is equal to the negative of the slope of this line; therefore, K_r for the MMP3/YZ103 complex is approximately 1×10^{-2} s^{-1} ($t_{1/2} = 69$ s). Also from eq 5, the intercept, b, of this line is equal to $\ln([C]_{eq}/[L]_0)$; thus, the equilibrium concentration of complex can be estimated from the intercept by

$$[C]_{eq} = [L]_0\, e^{b} \quad (9)$$

Substituting in the appropriate values for b and $[L]_0$ yields an estimated equilibrium concentration for the MMP3/YZ103 complex of 0.997 μM. The equilibrium concentrations of free ligand and protein in the affinity selection mixture can be calculated from the estimated value of $[C]_{eq}$ using eqs 6a

and 6b. Inserting these values and $[C]_{eq}$ into eq 2 yields an extrapolated estimate of the equilibrium binding affinity, K_D, of 9 nM. The value of the dissociation constant determined previously in an equilibrium measurement was 1 nM.

The equilibrium protein and complex concentrations calculated with eqs 6a and 6b are small values obtained by taking differences between relatively large values (for example, $[L]_{eq} = 1\ \mu M - 0.997\ \mu M = 0.003\ \mu M$), thus the relative uncertainties in these estimated concentrations are large. Taking the product of these estimated values to calculate K_D, eq 2, further magnifies the resultant uncertainties. Thus, relatively small errors in the extrapolated intercept of Figure 6 will lead to relatively large uncertainties in the estimated value of K_D. The data in Figure 6 represent the average values for three measurements; the error bars represent approximately one standard deviation in the measured values. The standard deviation in the measured response ratios, L/L_0, is approximately 8% which translates to an uncertainty in the extrapolated value for $\ln(L/L_0)$ of about 0.17. Propagating this uncertainty through eqs 9, 6a, and 6b, and then eq 2 ultimately leads to an uncertainty in K_D of approximately ± 500 nM.

The off-rate for the MMP3/YZ103 complex is calculated directly from the slope of the line represented in Figure 6. The uncertainty in the calculated value is directly proportional to the uncertainty in the measured slope. For these data the estimated off-rate and uncertainty are $1 \pm 0.2 \times 10^{-2}\ s^{-1}$ ($t_{1/2} = 69 \pm 11$ s). However, this calculation contains a systematic error arising from the assumption that the unbound ligand is immediately and completely removed from the proteinacous fraction during SEC isolation and therefore the dissociation time, t, is equal to the SEC retention time. In reality the concentration of unbound ligand is gradually reduced to nearly zero over a finite time. Therefore, the effective dissociation time is necessarily less than the SEC retention time, and the actual off-rate is necessarily faster than the calculated value. The off-rate of the MMP3/YZ103 complex has not been determined in an equilibrium measurement so we cannot estimate the magnitude of the error at this time but anticipate that the magnitude of the error will be relatively small.

Estimating Relative K_D Values. For a mixture of two (or more) ligands reacting reversibly with a protein

$$R + L_1 + L_2 + ... \rightleftharpoons C_1 + C_2 + ... \quad (10)$$

the equilibrium binding affinity, K_D, for any ligand in the mixture, L_i, is

$$K_{Di} = [R][L_i]/[C_i]_{eq} \quad (11)$$

By rewriting the equilibrium concentration of ligand, $[L_i]$, as the difference between the initial ligand concentration and the final complex concentration, eq 6a, the ratio of binding affinities for any pair of ligands in the mixture will be

$$K_{D1}/K_{D2} = ([C_2]_{eq}/[C_1]_{eq})([L_1]_0 - [C_1]_{eq})/([L_2]_0 - [C_2]_{eq}) \quad (12)$$

If the initial concentrations of the ligands are approximately equal and either the binding affinities for the two ligands

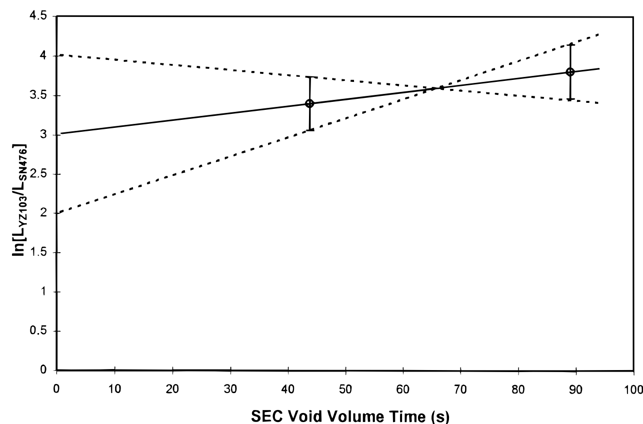

Figure 8. Estimation of relative binding affinities, YZ103 relative to SN476: log of the ratio of responses for the two affinity-selected ligands vs SEC void volume time. Data points represent the average values for three measurements; error bars represent one standard deviation.

are approximately equal or the initial concentration of the protein is much less than the initial concentrations of the ligands (so that the equilibrium concentration of ligand is approximately equal to the initial concentration), then eq 12 reduces to

$$K_{D1}/K_{D2} \approx [C_2]_{eq}/[C_1]_{eq} \quad (13)$$

The concentrations of both complexes will decrease exponentially due to dissociation during SEC isolation. The ratio of binding affinities expressed in terms of the measured mass spectrometric responses for the recovered complex becomes

$$K_{D1}/K_{D2} \approx (L_2/L_1)e^{(K_{r2} - K_{r1})t} \quad (14)$$

and, a linear relationship can be produced by taking the log of eq 14 and rearranging

$$\ln(L_2/L_1) \approx (K_{r1} - K_{r2})t + \ln(K_{D1}/K_{D2}) \quad (15)$$

A plot of the log of the ratio of the responses for the two ligands vs the retention time will give a straight line with a slope equal to the difference in the off-rates of the two ligands and an intercept equal to the log of the ratio of the K_D values. Figure 8 illustrates this relationship for the complexes of MMP3 with YZ103 and SN476 (equilibrium dissociation constants of 1 and 33 nM, respectively) formed under protein-limited affinity selection conditions. (Under these conditions the initial concentration of the protein is significantly less than those of the ligands so that the equilibrium concentrations of the ligands are approximately equal to the initial concentrations and the approximate relationship described in eq 13 is valid.) Using SN476 as the "reference", the value of K_D for YZ103 estimated from the intercept of the ratio of responses for the two ligands is 1.6 nM.

The data in Figure 8 are again the average values for three measurements with the error bars representing approximately one standard deviation. The uncertainty in the value of ln-(L/L_0) extrapolated to time zero in Figure 8 is approximately 1.0. This is significantly larger than the uncertainty in the

intercept for the estimation of the absolute binding affinity, Figure 6. But because the relative binding affinity is calculated directly from the intercept, the error is not magnified as it was in the absolute estimation of K_D. The uncertainty limits for the relative determination of K_D for YZ103 are 0.6−4.6 nM. The principal advantage in determining relative affinities is that the experimental uncertainties are not magnified as in the determination of absolute affinities. A possible disadvantage of the relative method is that it may have a more limited dynamic range. Because the ratio of measured responses, L_2/L_1, is inversely proportional to the ratio of binding affinities, K_{D1}/K_{D2}, the binding affinity ratio for reference and unknown ligands must, to first approximation, be less than the dynamic range of the mass spectrometric measurement. The linear response range of the method reported here was estimated to be approximately a factor of 50. Thus, using SN476 (with a binding affinity of 33 nM) as the reference limits the determination of unknown binding affinities to ligands with dissociation constants between about 0.7 nM and 1.6 μM.

If the off-rates for two protein/ligand complexes are approximately equal, then the exponential term in eq 14 approaches unity and the expression reduces to

$$K_{D1}/K_{D2} \approx [L_2]/[L_1] \qquad (16)$$

For the YZ103/SN476 pair the off-rates (as estimated from the slopes of $\ln(L/L_0)$ vs retention time plots for the two ligands) differ by about a factor of 3: 1×10^{-2} s^{-1} for YZ103 vs approximately 3×10^{-2} s^{-1} for SN476. At the shorter SEC isolation time represented in Figure 8 (void volume time = 43 s) the exponential term in eq 14 is about 2, thus we expect the binding affinity estimated form this single point to deviate from the extrapolated estimate by about a factor of 2. The relative binding affinity for YZ103 estimated from eq 16 using the $[L_2]/[L_1]$ ratio measured at 43 s retention time is 1.1 nM. Fortuitously, the systematic errors in measuring the response ratio have led to an estimated binding affinity nearer to the accepted value than that obtained with the extrapolation.

Analysis of Combinatorial Mixtures. To demonstrate the utility of this approach in a combinatorial leads discovery application, a 36-member combinatorial mixture was examined. The specific protocol for an analysis, of course, depends on the nature of the mixture being analyzed and the information desired. In this example we have the relatively simple situation of a small, well-characterized mixture of small molecules with minimal molecular weight redundancy which is known to be active for MMP3. We need to identify the library members with the highest binding affinities and estimate their relative dissociation constants. The protocol was (1) incubate mixture with MMP3 under protein limited conditions; (2) perform the online SEC/LC-MS analysis to determine the identities of the "active" components from their molecular weights; and (3) perform the positive control (the total quantity of each ligand analyzed was approximately 10 pmol, no target protein present) to determine the response factors for calculating relative dissociation constants. For this demonstration the negative control was also run to confirm

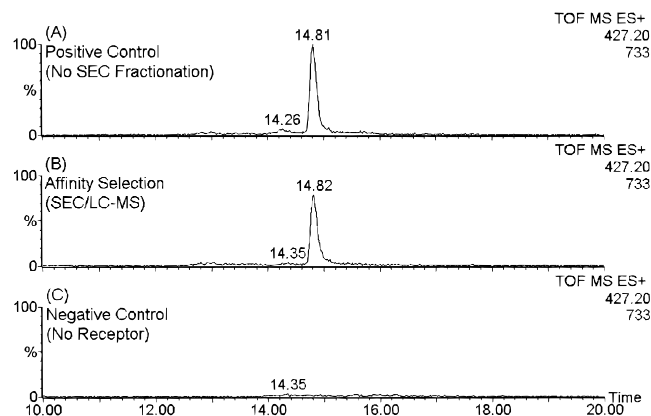

Figure 9. Selected ion traces for an active member of a combinatorial mixture, $[M + H]^+$ at m/z 427: (A) positive control, (B) affinity-selected sample, (C) negative control.

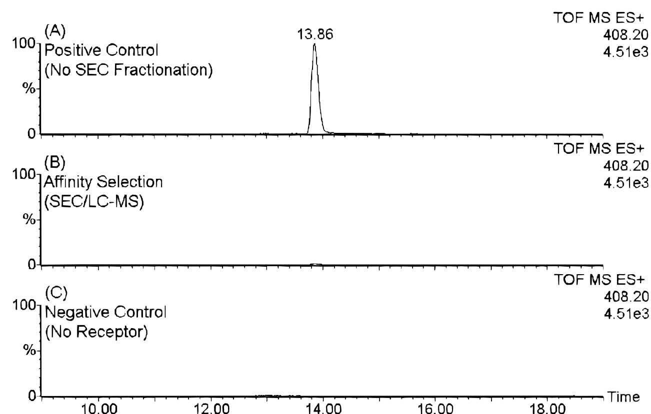

Figure 10. Selected ion traces for an inactive member of a combinatorial mixture, $[M + H]^+$ at m/z 408: (A) positive control, (B) affinity-selected sample, (C) negative control.

the separation of the proteinacous fraction from the free library.

Figures 9 and 10 show the results for an active library member with $[M + H]^+$ at m/z 427 and an inactive member, $[M + H]^+$ at m/z 408. Neither component is observed in the negative control, Figures 9C and 10C, confirming that the unbound components will not interfer with the analysis of the affinity-selected sample. None of the 36 components were detected in the negative control. Figures 9A and 10A show that both components are observed in the positive control. All 36 library members were observed in the positive control. In the SEC/LC-MS analysis of the affinity-selected sample the m/z 427 component is observed, Figure 9B, and the m/z 408 component is not detected, Figure 10B. Qualitatively, the analysis of the affinity-selected sample has established that the m/z 427 component is tightly bound to MMP3 while the m/z 408 component is not.

Four active members were determined in the affinity selection analysis of the mixture. Table 2 summarizes the data and the estimated relative binding affinities. Discrete resynthesis and conventional screening of the active components confirmed the rank order of the relative binding affinities determined by affinity selection/SEC/LC-MS. A limited number of the components not identified as active by the affinity selection/SEC/LC-MS analysis were resynthesized as discretes and screened to confirm the negative result.

Table 2

		integrated responses				
m/z	LC-MS RT[a]	negative control	positive control (L_0)[b]	affinity-selected (L)[b]	fractional recovery (L/L_0)	relative dissociation constant
331.2	8.10	0	210.9	135.6	0.643	0.73
365.2	8.19	0	205.3	87.3	0.425	0.48
427.2	8.56	0	99.8	87.6	0.878	1.00
466.3	8.10	0	230.5	34.8	0.151	0.17

[a] Retention time in minutes. [b] Area under selected ion trace for $[M + H]^+$, arbitrary units.

Conclusions

Online SEC/LC-MS analysis of affinity-selected ligands is an effective means for determining the members of mixtures which form tightly bound noncovalent complexes with target proteins. Affinity-selected ligands can be determined and relative binding affinities estimated using 20 pmol (or less) of the target protein.

Measured SEC recovery efficiencies for ligands known to form tight noncovalent complexes with the target protein were relatively low and depended on the SEC isolation time (as governed by the flow rate). A kinetic analysis indicated that the decrease in recovery efficiency is consistent with unimolecular dissociation of the complex during SEC isolation. Linking the kinetic results with equations describing the equilibrium complex concentrations shows that the SEC/LC-MS response for an affinity-selected ligand depends on the binding affinity of the ligand, the off-rate of the complex, and the SEC isolation time. This analysis is useful for establishing both the binding affinity and the off-rate limits, for detecting affinity-selected ligands under specific experimental conditions, and for ascertaining the conditions needed to detect ligands with specific binding affinities and/or off-rates.

The user controllable parameter effecting the complex recovery efficiency is the SEC isolation time. The online SEC/LC-MS system described here uses short BioSep silica packed SEC columns which can withstand relatively high flow rates. The higher flow rates combined with the short length of the columns significantly reduces the SEC isolation time thereby decreasing the extent of complex dissociation and increasing the sensitivity of the online SEC/LC-MS analysis. For the target protein and ligand mixtures reported here it was possible to obtain satisfactory SEC resolution of complex and ligands while achieving isolation times of less than 1 min. The BioSep SEC packing did, however, appear to exhibit nonspecific binding with both the ligands and the target protein under the elution conditions reported here. Further evaluation is needed to determine if this is problematic.

Two methods for extrapolating equilibrium binding affinities and off-rates from the measured nonequilibrium distributions were derived from the kinetic analysis of the SEC isolation process. The principal drawback in determining absolute binding affinities is that the calculation magnifies errors in the measured distributions. Uncertainties on the order of ±500 nM were encountered in the determination for a ligand with a binding affinity of 1 nM. The uncertaines encountered in estimating relative equilibrium binding affinities were smaller, on the order of ±2 nM in the determination of the binding affinity of the same 1 nM ligand. In the case of protein/ligand complexes with slow off-rates or nearly equal off-rates, it may be possible to rank relative binding affinities of detected components without using an extrapolation (i.e., from a single time point).

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