Published in final edited form as:

Comb Chem High Throughput Screen. 2008 January ; 11(1): 1–6.

Development of a screening assay for ligands to the estrogen receptor based on magnetic microparticles and LC-MS

Yongsoo Choi and Richard B. van Breemen*

UIC/NIH Center for Botanical Dietary Supplements Research, Department of Medicinal Chemistry and Pharmacognosy, University of Illinois College of Pharmacy, 833 South Wood Street, Chicago, IL 60612 USA

Abstract

A high throughput screening assay for the identification of ligands to pharmacologically significant receptors was developed based on magnetic particles containing immobilized receptors followed by liquid chromatography—mass spectrometry (LC-MS). This assay is suitable for the screening of complex mixtures such as botanical extracts. For proof-of-principle, estrogen receptor- α (ER- α) and ER- β were immobilized on magnetic particles functionalized with aldehyde or carboxylic acid groups. Alternatively, biotinylated ER was immobilized onto streptavidin-derivatized magnetic particles. The ER that was immobilized using the streptavidin-biotin chemistry showed higher activity than that immobilized on aldehyde or carboxylic acid functionalized magnetic particles. Immobilized ER was incubated with extracts of *Trifolium pratense* L. (red clover) or *Humulus lupulus* L. (hops). As a control for non-specific binding, each botanical extract was incubated with magnetic particles containing no ER. After magnetic separation of the particles containing bound ligands from the unbound components in the extract, the particles were washed, ligands were released using methanol, and then the ligands were identified using LC-MS. The estrogens genistein and daidzein were identified in the red clover extract, and the estrogen 8-prenylnaringenin was identified in the hop extract. These screening results are consistent with those obtained using previous screening approaches.

Introduction

Although cell-based assays may be used to test compounds for functional activity [1], receptor binding [2], cell membrane permeability [3] and even cytotoxicity [4], these assays are slow and provide no structural information regarding the active compounds that might be present, which is a disadvantage when mixtures are screened. Alternatively, high-throughput screening may be carried out using an isolated receptor or enzyme based on the interaction of pharmacologically active compounds with specific macromolecular targets [5]. Most screening assays of this type have been combined with optical detection, such as UV absorbance or fluorescence detection, because of its high sensitivity and speed. However, optical detection can suffer from matrix interference, such as light scattering and/or absorption by matrix, especially when complex samples are analyzed. Furthermore, slow and labor intensive steps are required to identify lead compounds in complex mixture such as botanical extracts.

Since mass spectrometry provides excellent selectivity, sensitivity, and structural information to facilitate the identification of ligands in complex mixtures, various screening assays have been developed based on multidimensional chromatography mass spectrometry, such as size-exclusion chromatography LC-MS [6], ultrafiltration LC-MS [7], affinity capillary

*To whom correspondence should be addressed: Tel: (312) 996-9353 Fax: (312) 996-7107 Breemen@uic.edu.

electrophoresis-MS [8], H/D exchange MALDI MS [9], and FT-ICR MS [10]. For example, we have reported the development of ultrafiltration LC-MS to screen mixtures of compounds for ligands to various receptors, such as estrogen receptors (ER) [11], cyclooxygenase—2 [12] and human serum albumin [13]. The ultrafiltration step facilitates the separation of ligand-receptor complexes from unbound compounds, and then LC-MS is used to characterize and identify the ligands.

In this paper, we describe a new screening assay which, like ultrafiltration LC-MS, is compatible with a wide range of binding buffers, prevents introduction of macromolecular targets or unbound compounds into the LC-MS system, and is fast and inexpensive. This new assay utilizes a separation step based on the binding of receptors to magnetic nano/micro particles followed by LC-MS identification of the released ligands. Magnetic particles and nanoparticles have been utilized for various biological applications, such as protein purification from cells [14], target cell extraction from tissues [15], targeting antibody detection [16], and gene analysis [17]. To the best of our knowledge, there have been no reports of the application of magnetic particles for screening of small molecular ligands to a biological receptor.

Because of the large surface area of nano/micro particles and well developed coupling chemistry, target receptors can be immobilized to these particles with high efficiency. After the ligands contained in a mixture have become bound to the immobilized receptors, the particles can be recovered magnetically from the solution in < 1 min. Then, LC-MS can be used to identify the ligands following their release from the magnetic particles. In this investigation, estrogen receptors (ERs) were immobilized onto chemically functionalized magnetic particles to demonstrate the utility of this screening method. The ERs retained their biological activity after immobilization and were useful for high throughput screening of complex botanical extracts for estrogenic ligands.

Experimental Methods

Materials

Magnetic particles (1 μm diameter) functionalized with aldehyde or carboxylic acid groups or containing immobilized streptavidin were purchased from Bioclone (San Diego, CA). Human recombinant estrogen receptors, ER— α and ER— β , were purchased from Invitrogen (Madison, WI). The isoflavones genistein and daidzein were purchased from Indofine Chemical (Hillsborough, NJ), and the coupling reagents, NaCNBH_3 and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were obtained from Sigma-Aldrich (St. Louis, MO). Sulfo-*N*-hydroxysuccinimide biotin ester, bicinchoninic acid (BCA) protein assay kit, and dialysis units (10,000 molecular weight cutoff) were purchased from Pierce (Rockford, IL). Centrifugal ultrafiltration filters (Microcon YM-30) were purchased from Millipore (Bedford, MA). Ethanolic extracts of the aerial parts of red clover, *Trifolium pratense* L., and hop cones, from *Humulus lupulus* L., were prepared as described previously [18].

Immobilization of receptors on magnetic particles

To optimize protein concentration, ionic strength and pH for ER immobilization, ER— α (as received from the vendor) was washed 5 times with 200 μL coupling buffer to remove storage buffer using ultrafiltration through a Microcon YM-30 30,000 molecular weight cut-off regenerated cellulose membrane. The coupling buffer (pH 5.5, 7.5 or 9.0) consisted of 15 mM HEPES or 15 mM HEPES containing 0.1 M NaCl and 10% glycerol. Magnetic particles (0.6 mg) functionalized with either aldehyde or carboxylic acid groups were pre-washed 3 times with 200 μL portions of coupling buffer and then mixed with different concentrations of ER— α ranging from 0.03 to 0.4 mg/mL at pH 7.5. The immobilization of ER— α on aldehyde functionalized magnetic particles occurred through the formation of Schiff's bases with protein

primary amines and was made irreversible by reduction by NaCNBH₃ for 14 h [19]. Carboxylic acid functionalized magnetic particles were activated by EDC for immobilization of protein amino groups through the formation of amide bonds, which was carried out during incubation with ER for 20 h [20]. A 250-fold excess of EDC or NaCNBH₃ was used relative to the ER, and the reactions were carried out in 1.5 mL Eppendorf tubes using gentle rotation.

The coupling efficiency between ER and the derivatized magnetic particles was determined based on the amount of protein remaining in solution after immobilization. A Pierce BCA protein assay kit was used to determine the protein concentrations. Aliquots (5 µL) of each reaction solution before and after immobilization were placed into separate wells of a 96 well plate, mixed with 200 µL BCA working reagent (as described by the manufacturer) and incubated at 37 °C for 30 min. The absorbance of each solution was measured at 562 nm using a microplate scanning spectrophotometer (Power Wave 200, Bio-Tek Instruments, Winooski, VT), and the coupling efficiency was calculated using the following equation:

$$\text{Coupling efficiency (\%)} = \frac{(A_{\text{before reaction-blank}}) - (A_{\text{after reaction-blank}})}{(A_{\text{before reaction-blank}})} \times 100$$

Based on these optimization experiments (results are shown in the next section), subsequent immobilization procedures consisted of the incubation of 700 pmol of ER with 1.2 mg of aldehyde or carboxylic acid functionalized magnetic particles in coupling buffer at pH 7.5 in a total volume of 100 µL. Although the pH could be as high as 9, pH 7.5 was used since it is closer to the physiological value.

As an alternative to covalent immobilization, biotinylated ER-β was immobilized onto streptavidin-derivatized magnetic particles as follows. Using a Pierce dialysis unit with a 10,000 molecular weight cut-off, 700 pmol of ER-β was dialyzed for 5 h against 1.0 L of PBS buffer (0.1 M, pH 7.4) containing 0.15 M NaCl and 10% glycerol. Then, ER-β was labeled with biotin by reaction for 1 h with a 20-fold molar excess of sulfo-*N*-hydroxysuccinimide biotin ester. The solution containing biotinylated ER was dialyzed for 2 h in the PBS buffer to remove unreacted biotin, and then the biotin labeled ER-β was incubated with 1.4 mg of a streptavidin immobilized magnetic particles for 1 h at room temperature with slow rotation. After incubation, the solution was washed with 200 µL of PBS buffer three times and stored at 4 °C until use.

Determination of activity of immobilized ER and screening for estrogens

The magnetic particles (0.6 mg aldehyde and carboxylic acid functionalized particles or 0.3 mg of streptavidin derivatized particles) containing immobilized ER-α or ER-β (300 pmol) were washed 3 times with 200 µL portions of Tris buffer (50 mM, pH 7.5) containing 0.1 M NaCl, 10% glycerol, and 1 mM EDTA and then incubated for 2 h at room temperature with genistein (0.667 µM) or botanical extract (100 µg/mL red clover extract or 40 µg/mL hop extract) in a total volume of 150 µL in a 1.5 mL Eppendorf tube containing Tris buffer. After incubation, each tube was placed into a magnetic separator for 1 min, and the solution was removed using a pipette. The magnetic particles containing immobilized ER and ligands were washed with 150 µL ammonium acetate buffer (35 mM, pH 7.5) by pipetting several times to remove unbound compounds. The washing step was repeated three times, and the ligand was released by adding 60 µL of methanol/water (60:40; v/v). The methanol/water solution was removed, mixed with 1 µL of naringenin (10 µM) as an internal standard, and analyzed for estrogens using liquid chromatography-mass spectrometry (LC-MS) as described below. Identical incubations using magnetic particles containing no ER were used to control for non-specific binding to the magnetic particles.

LC-MS analysis

Aliquots of each ligand solution (60 μ L) were analyzed using LC-MS with either an Agilent (Palo Alto, CA) G1946C single quadrupole mass spectrometer (for the quantitative analysis of genistein) equipped with a model 1100 HPLC system or a Micromass (Manchester, UK) QTOF2 hybrid mass spectrometer (for screening) equipped with a Waters (Milford, MA) 2690 HPLC system. Separations were carried out using a Waters XTerra HPLC column (2.1 mm $\times$ 150 mm, 3.5 μ m particle size) with a solvent system consisting of a 15 min linear gradient from 30 - 65% aqueous acetonitrile at 2.0 mL/min (for genistein analysis) or a 25 min linear gradient from 30 - 80% acetonitrile at 0.2 mL/min. The HPLC column was re-equilibrated at 30% acetonitrile for at least 15 min between injections. For screening, negative ion electrospray mass spectra were recorded over the range m/z 200 - 600 in 1 s. For quantitative analyses, selected ion monitoring was used to record the signals for the deprotonated molecules of genistein and the internal standard at m/z 269 and 271, respectively, with a dwell time of 0.5 s/ion.

Results and Discussion

Assay development and optimization

A scheme summarizing the design of the magnetic bead assay and the steps used in the screening of mixtures for ligands to the ER is shown in Fig. (1). First, a solution containing potential estrogens is incubated with ER immobilized on magnetic particles, and then the ligand-receptor complexes are isolated magnetically. After washing to remove residual unbound compounds, the ligands are released by treatment with 60% aqueous methanol and then characterized using LC-MS. To control for non-specific binding to the magnetic particles, a duplicate incubation and analysis is carried in which the immobilized ER is replaced with magnetic particles containing no protein. Comparison of the LC-MS chromatograms of the experiment and control show signal enhancement for the peaks corresponding to ligands of the ER.

Using the estrogenic botanical isoflavone genistein as a test ligand for the new screening assay, the experimental conditions for the immobilization of ER- α and ER- β were optimized. First, the optimum pH for ER- α immobilization on aldehyde functionalized magnetic particles was determined as shown in Fig. (2). Immobilization at pH 9.0 produced ER- α with the highest activity followed closely by immobilization at pH 7.4. No activity was detected following immobilization at pH 5.5, since the peak area of genistein did not increase relative to the control incubation. As shown in Table 1, similar pH effects were observed for the immobilization of ER- β on aldehyde functionalized magnetic particles.

Next, pH optimization experiments were carried for the immobilization of ER- α on carboxylic acid functionalized magnetic particles, and the results are summarized in Table 1. Like the aldehyde functionalized magnetic particle experiments, the highest ER activity on carboxylic acid functionalized magnetic particles was detected after immobilization at pH 9.0 followed by pH 7.4, and no activity was detected when pH 5.5 was used for immobilization. Since the extent of immobilization (indicated as coupling efficiency in Table 1) was actually highest at pH 5.5 whether using aldehyde or carboxylic acid functionalized magnetic particles, the lack of ER activity at pH 5.5 was probably the result of protein denaturation.

The role of ionic strength of the immobilization buffer on the activity of ER- α and the combination of varying both ionic strength and pH were investigated. The results are also summarized in Table 1. Unlike pH, the concentration of NaCl in the buffer had no significant effect on ER activity whether immobilized on aldehyde or carboxylic acid functionalized magnetic particles.

The effect of ER concentration on immobilization efficiency was investigated using aldehyde functionalized magnetic particles, and the results are shown in Fig. (3). Over the concentration range studied (up to 400 $\mu\text{g/mL}$), the efficiency of immobilization actually increased as concentration increased. For example, the amount of ER- α immobilized on the aldehyde functionalized magnetic particles increased ~11-fold as the initial concentration of ER- α was increased from 30 to 400 $\mu\text{g/mL}$. This observation is consistent with the results of previous studies [20,21] and was probably due to protein-protein coupling in addition to protein-magnetic particle coupling. Additional experiments were carried out using a 600-fold molar excess of coupling reagents (data not shown). Although 100% coupling efficiency could be achieved in 5 h using a 600-fold molar excess of coupling reagents, the immobilized ER did not show any activity, presumably due to protein-protein cross-linking. Instead, optimum activity was obtained when the coupling efficiency was in the range of 40-50% using pH 7.5 to 9.0 and the conditions described in Table 1.

Although covalent attachment of ERs to aldehyde or carboxylic acid functionalized magnetic particles could result in active receptor suitable for screening mixtures, this approach required optimization of the immobilization conditions and was a compromise between immobilization efficiency and activity. As a potentially more efficient alternative, immobilization of biotin tagged ER onto streptavidin derivatized magnetic particles was investigated. After reaction with a biotin ester, biotinylated ER- β (700 pmol) was bound non-covalently to streptavidin derivatized magnetic particles (1.4 mg) in 1 h. Note that the immobilization time was reduced significantly using this affinity approach compared to the 14 h required for covalent attachment to aldehyde or carboxylic acid functionalized magnetic particles. Furthermore, 95% coupling efficiency was obtained under these conditions compared to 40-50% using covalent attachment of active ER. As shown in the screening data in Figs (4-6), the high coupling efficiency of the affinity immobilization approach enabled smaller quantities of magnetic particles to be used per screening assay (0.3 mg) than when using ER immobilized on aldehyde or carboxylic acid functionalized particles (0.6 mg).

Screening of botanical extracts

Red clover and hop extracts were screened for ligands to ER- β that had been immobilized on magnetic particles using each of the three methods in order to evaluate the new screening assay. First, either aldehyde or carboxylic acid functionalized magnetic particles containing immobilized ER- β were incubated with a red clover extract containing estrogenic isoflavones. After washing to remove unbound compounds and then releasing the bound compounds, the ligands were analyzed using LC-MS. The LC-MS chromatograms for these assays and the corresponding controls are shown in Fig. (4). Based on the increases in peak areas in the experimental incubations compared to the controls, the compounds with the highest affinities for ER- β in red clover were daidzein and genistein (detected as their deprotonated molecules of m/z 253 and at m/z 269, respectively, during negative ion electrospray LC-MS). Also detected were the much more abundant but much less estrogenic isoflavones formononetin (m/z 267) and biochanin A (m/z 283). These isoflavones were identified by comparison to standards. These results are consistent with our previous ultrafiltration LC-MS screening study of an ethanolic extract of red clover [2].

The signal-to-noise ratio for the assay using the aldehyde functionalized magnetic particles was slightly superior to that obtained when using a comparable amount of the carboxylic acid functionalized particles (see Fig. (4)). These results suggest that the activity of ER- β was higher when immobilized on the aldehyde functionalized magnetic particles. Regardless of which immobilization method was used, no false positives were detected, and there was no interference from the complex botanical matrix.

Since ER- β immobilized on aldehyde functionalized magnetic particles provided better signal-to-noise during screening of a red clover extract than did the carboxylic acid, ER- β immobilized on aldehyde functionalized magnetic particles was used to screen a hop extract. As shown in Fig. (5), a compound in the hop extract was detected eluting at 22.4 min that showed high affinity for ER- β . Based on comparison with standards, this ligand was identified as 8-prenylnaringenin. In addition to 8-prenylnaringenin, peaks for the related but non-estrogenic or weakly estrogenic compounds isoxanthohumol, xanthohumol and 6-prenylnaringenin are indicated in Fig. (5). These results are consistent with previously reported screening studies of hop extracts [18], which found that 8-prenylnaringenin was the most potent estrogen in hop extracts.

Although the data in Figs. (4) and (5) show that covalent immobilization of ER onto functionalized magnetic particles may be used with LC-MS to screen botanical extracts for estrogens, the immobilization methods used could not achieve a high density of protein coverage on the particles without loss of ER activity. To achieve higher activity for the immobilized ER, the high affinity between biotin and streptavidin was used to immobilize biotinylated ER- β onto streptavidin derivatized magnetic particles. Using only 0.3 mg of affinity-immobilized ER- β instead of 0.6 mg as in the assays with covalently immobilized ER- β , the hop extract was reanalyzed, and the LC-MS screening results are shown in Fig. (6). Similar to the LC-MS chromatograms in Fig. (5), the signal for 8-prenylnaringenin at a retention time of 22.4 min was enhanced in the experiment compared to the control containing particles without ER- β , and no signal enhancement was detected for isoxanthohumol, 6-prenylnaringenin or xanthohumol. Therefore, by utilizing biotin/streptavidin affinity for the immobilization of ER- β , the amount of magnetic particles used per assay could be reduced by 50%.

Conclusions

Active ERs could be immobilized on aldehyde or carboxylic acid functionalized magnetic particles through coupling reactions mediated by NaCNBH₃ or EDC, respectively, or through avidin/streptavidin affinity methods, and then used in combination with LC-MS for screening natural product mixtures such as botanical extracts for estrogens. Although almost complete immobilization of all solution-phase ER could be obtained through covalent reaction, extensive immobilization resulted in complete loss of ER activity. Therefore, optimization experiments were required to maximize the activity of covalently immobilized ER for screening. Since the optimum parameters for the immobilization of active ER might be different for other proteins, additional optimization experiments might be required for each new receptor protein used for screening. As a convenient alternative to covalent immobilization that did not require optimization of the reaction conditions, ER could be biotinylated and immobilized in active form on streptavidin derivatized magnetic particles with 95% efficiency. Therefore, the use of affinity methods for receptor immobilization offers advantages over the covalent immobilization approach.

The use of magnetic particles containing immobilized ER facilitated the rapid isolation of the ER-ligand complexes from botanical extracts. It was hoped that the use of these particles would reduce or eliminate non-specific binding compared to other approaches such as pulsed ultrafiltration LC-MS [22], but the background signals were similar. Like pulsed ultrafiltration LC-MS, this new method should be applicable to the screening of other receptors besides ERs. In conclusion, the use of receptors immobilized on magnetic particles in combination with LC-MS offers a new approach for the rapid screening of mixtures such as combinatorial libraries or botanical extracts for drug discovery.

Acknowledgements

This research was supported by grant P50 AT00155 provided to the UIC/NIH Center for Botanical Dietary Supplements Research by the Office of Dietary Supplements, the Office for Research on Women's Health, and the National Center for Complementary and Alternative Medicine. The content is the responsibility of the authors and does not necessarily represent the official views of the sponsors.

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Fig. (1).

Scheme showing the magnetic particle LC-MS screening assay for ligands to the estrogen receptor (ER). Magnetic particles containing a) immobilized ER; or b) no receptor (control) are incubated with a mixture such as a combinatorial library or natural product extract for 2 h in Tris buffer at pH 7.5. By applying a magnetic field to the sample tubes, the magnetic particles are removed from the incubation buffer. After washing the magnetic particles to remove unbound compounds, the ligands are released from the receptor with 60 μ L of aqueous methanol (60%) and characterized using LC-MS.

Fig. (2).

Effect of immobilization pH on the activity of ER- α attached to aldehyde functionalized magnetic particles. Negative ion electrospray LC-MS chromatograms, normalized to the signal for the internal standard naringenin (m/z 271), indicate the relative amounts of genistein (m/z 269) that had been bound to ER- α . The control assay was identical except that the magnetic particles contained no ER. For each experiment, 300 pmol of ER- α was reacted with 0.6 mg aldehyde functionalized magnetic particles in 60 μ L coupling buffer at the pH indicated. After incubation with 0.667 μ M genistein, genistein was released and analyzed using LC-MS. Immobilization on aldehyde functionalized magnetic particles at pH 9.0 produced ER- α with the highest activity.

Fig. (3).

The effect of initial ER- α concentration on coupling efficiency with aldehyde functionalized magnetic particles. ER- α was immobilized on 0.6 mg of magnetic particles in 60 μ L coupling buffer at pH 7.5 containing a 250-fold excess of NaCNBH₃. Each reaction was carried out for 14 h at room temperature.

Fig. (4).

Negative ion electrospray LC-MS chromatograms showing the screening results for ligands to ER-β in an ethanolic extract of the aerial parts of red clover. A) ER-β was immobilized on aldehyde functionalized magnetic particles (0.6 mg); and B) ER-β was immobilized on carboxylic acid functionalized magnetic particles. The solid line represents screens using immobilized ER-β, and the dotted line represents the control incubation using magnetic particles without protein. These computer-reconstructed mass chromatograms show the signals for the deprotonated molecules of the estrogenic isoflavones daidzein (m/z 253) and genistein (m/z 269) and their methylated analogues, formononetin (m/z 267) and biochanin A (m/z 283).

Fig. (5).

Negative ion electrospray LC-MS screening of a hop extract for ligands to ER-β immobilized on aldehyde functionalized magnetic particles. The solid line represents incubation with the hop extract and magnetic particles (0.6 mg) containing immobilized ER-β; and the dotted line represents a control incubation that was identical except that the particles contained no protein.

Fig. (6).

Screening of a hop extract for ligands to ER- β using biotinylated ER- β immobilized onto streptavidin functionalized magnetic particles (0.3 mg). The solid line represents incubation with the hop extract and magnetic particles containing immobilized ER- β ; and the dotted line represents a control incubation that was identical except that the particles contained no protein. Among the most estrogenic botanical products, 8-prenylnaringenin was detected in the extract at a retention time of 22.4 min.

Table 1

Coupling efficiency and activity of ER- α (300 pmol) reacted for 14 h with aldehyde or carboxylic acid functionalized magnetic particles (0.6 mg) in 60 μ L coupling buffer and a 250-fold molar excess of NaCNBH₃ or EDC.

Conditions		Coupling efficiency		LC-MS peak enhancement (fold increase relative to control) ^a	
pH	NaCl ^b	Aldehyde	Carboxyl	Aldehyde	Carboxyl
5.5	-	55 %	67 %	0	0
	0.1 M	52 %	- ^c	0	0
7.5	-	42 %	54 %	3.9	3.4
	0.1 M	43 %	-	4.1	3.9
9.0	-	42 %	56 %	4.3	4.0
	0.1 M	45 %	-	4.6	4.1

^aER activity was based on genistein binding relative to control as measured using LC-MS.

^bTo investigate the effect of increased ionic strength on ER immobilization, 0.1 M NaCl and 10% glycerol were added to the 15 mM HEPES buffer solution.

^cAddition of 0.1 M NaCl and 10 % glycerol interfered with the protein concentration absorbance assay.