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Desorption/Ionization on Mesoporous silicate (DIOM)-Mass Spectrometry (MS) and Ultrafiltration for Target-based Drug Screening

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Abstract

This study presents a novel approach for rapid screening of combinatorial libraries to identify binding molecules for target proteins. Ultrafiltration coupled with desorption/ionization on mesoporous silicate (DIOM)-mass spectrometry (MS) provides a rapid, simple but powerful approach for the preliminary screening of active ligands toward specific target receptors. The feasibility of this strategy is demonstrated with a library of small molecule ligands (11 compounds) and the target protein, human serum albumin (HSA). Equimolar mixture of library was incubated with HSA under native binding conditions. Subsequently, the high molecular weight protein-drug complexes were selectively compartmented by ultrafiltration. The bound small molecular weight drugs were released and then recovered by denaturation of HSA for analysis by DIOM-MS. DIOM -MS directly provided clean mass spectra of binding compounds without any interference below 300 Da. This simple and robust method can preliminarily screen various chemical entities from a combinatorial mixture to find compounds with strong binding affinity (above 1.0×10^3 M^{-1}) toward the target protein.

Keywords: DIOM-MS, Ultrafiltration, Drug screening, HSA

Introduction

In classical drug discovery, drug candidates are sequentially designed, synthesized, purified, characterized, screened and investigated for biological activity one by one[1,2]. Recently, big advances in the synthesis of chemicals in combinatorial libraries have led to the ability to rapidly find lead compounds. However, the critical issue has been screening these libraries to select molecules with a desired biological activity or binding property that is specific for a given target, such as enzyme, DNA, antibody, or antigen, because most classical screening assays test compounds singly[3]. Several methods allow receptors and ligands to be screened in solution so as to preserve their intact binding interactions, and multiple assays can be carried out in parallel in an approach called high-throughput screening[4-7]. Target-based drug screening is an especially important strategy for new drug development[8]. Two general methods for target-based screening are typically used. One is function-based screening, in which modulation of the biological activity of the target by the compounds is investigated[4]. The other is an affinity-based screening that takes advantage of the binding affinities of the combinatorial library for targets, such as immobilized ligands or immobilized receptors, to screen for potential lead compounds[6]. However, affinity-based methods present difficulties in that immobilization of the target may change the affinity characteristics of the bound compounds versus that of the native, solution-phase form.

Protein-drug binding is an important event in biosystems because it can influence the absorption, distribution, metabolism, and excretion (ADME) properties of typical drugs[9,10]. Human serum albumin (HSA) is the most abundant protein in the circulation; plasma is composed of 60% HSA. Thus, HSA-drug binding can inhibit a drug's ability to reach therapeutic levels and decrease its efficiency as a viable drug[10]. A drug with high HSA binding affinity will have a long half-life, which may increase its toxicity in the human body. On the other hand, a drug with low binding affinity has a disadvantage in its ability to perfuse tissues and reach the site of action. Therefore, knowing the appropriate and precise therapeutic

Figure 1. Image of deposited mesoporous silicate with sample on MALDI target plate. (a) SEM image of Al-SBA-15, (b) Photograph of deposited Al-SBA-15 with samples on MALDI target plate.

level and characterizing the extent of drug binding are prerequisites for the desired effect[10].

Several methods, including equilibrium dialysis[11], ultrafiltration[12], LC/MS[13], and CE/MS[14], have previously been presented to screen ligands against proteins. Although these approaches can provide precise information, they are time-consuming and require long incubation times. Thus, these cannot provide rapid screening methodology. In addition, many studies have been performed to analyze the binding affinity of immobilized receptors. Although binding constants can be elucidated from immobilize receptors, the restricted condition of the bound receptors decreases the inherent properties of HSA and immobilized HSA columns are expensive[15].

Mass spectrometry (MS) has been used in drug discovery for decades. Typically, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), as soft ionization methods, are useful in the analysis of target identification, characterization, structural identification, drug metabolism, and pharmacokinetics in drug discovery[2,3]. Because MALDI-MS has high sensitivity, high salt tolerance, and can be automated for high-throughput analysis, it is especially well suited as the first screening methodology in drug discovery. However, a variety of different compounds such as organic acids, amino acids, and synthetic drugs are difficult to analyze by conventional MALDI-MS due to the inherent noise below 500 Da generated by organic matrixes of low molecular weight[16]. During ionization by laser irradiation, matrixes act as the matrix for themselves and produce many noise signals, which can even suppress the sample peaks[17].

To solve the above issues in drug screening, we present a solution-based affinity selection methodology combined with previously developed desorption/ionization on mesoporous silicates (DIOM)-mass spectrometry (MS) to rapidly screen binding compounds against HSA. Our proposed method requires minimal sample preparation and provides analysis of the binding event in a high-throughput manner with mass spectrometry. The combination of ultrafiltration and DIOM-MS facilitates the screening of ligands that bind with affinity to specific receptors in solution.

Results and Discussion

Our previous study suggested that DIOM-MS might be useful for large-scale drug screening in which most of the lead compounds are small molecules and small peptides[17]. In the analysis of the mixture, no noise signals that interfered with the analytes were ever detected in the DIOM-MS spectrum, although relatively higher laser intensities (45-55%) were used; this is a reflection of the inert property of the ordered mesoporous material during laser irradiation. In this study, DIOM-MS combined with ultrafiltration was evaluated for drug screening.

Drug screening generally involves binding assays to determine the affinity of drug candidates for a particular binding target, such as a protein. Thus, solution phase binding assays, in which drug candidates form tightly bound complexes with the target protein, are the most desirable for screening molecules of high specificity. A schematic diagram of the overall procedure for drug screening is presented in Figure 2. To validate this application for drug screening, a binding assay was performed by incubating

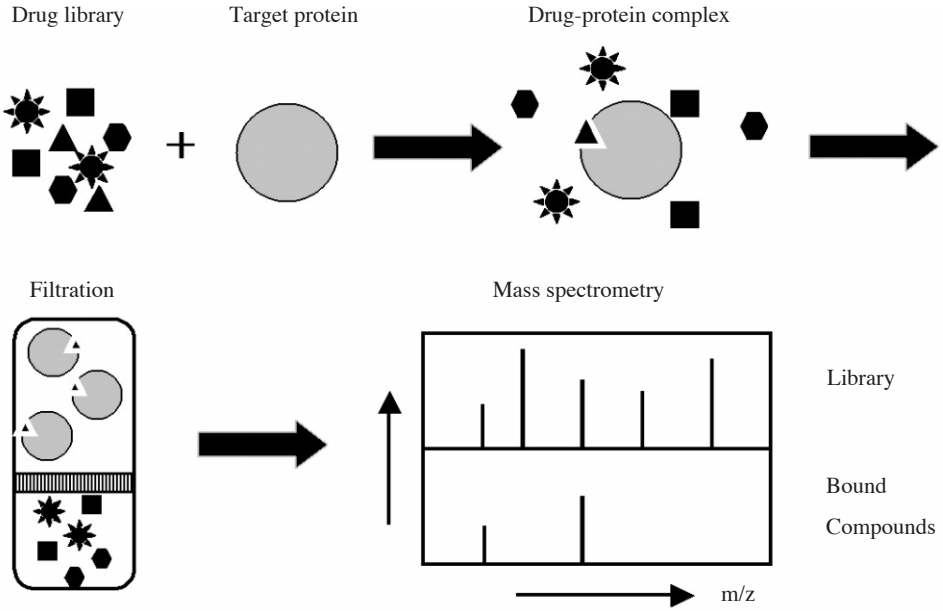

Figure 2. Schematic diagram of screening method using DIOM-MS/ Ultrafiltration; Target protein, HSA, was incubated with a library under native conditions in buffer solution. Incubation was stopped after 15 min, which was enough time for HSA to bind with any compounds listed in Table 1.

Table 1. Drug library for binding to human serum albumin (HSA).

Number	Name	Calculated Mass $[M+H]^+$ [a]	Detected Mass[b]
1	GABA[c]	104.07	142.04 $[M+K]^+$
2	Ornitine	133.10	171.07 $[M+K]^+$
3	Arginine	175.12	175.18 $[M+H]^+$
4	Histidine	156.08	194.02 $[M+K]^+$
5	Naproxen[d]	230.09	230.20 $[M]^+$
6	Tryptophan	205.10	243.07 $[M+K]^+$
7	Kynurenine	209.09	247.06 $[M+K]^+$
8	Alprenolol	250.18	250.27 $[M+H]^+$
9	Propranolol	260.17	260.25 $[M+H]^+$
10	Imipramine	281.20	281.19 $[M+H]^+$
11	Warfarin	309.11	347.02 $[M+K]^+$

[a]Calculated mass: monoisotope molecular weight was calculated from MolE (Molecular Mass Calculator v2.0; http://medlib.med.utah.edu/masspec/mole.htm)
[b]Detected m/z: all peaks were detected as $[M+H]^+$ or $[M+K]^+$.
[c]GABA: γ-aminobutyric acid
[d]Naproxen was detected as $[M]^+$ form.

human serum albumin (HSA; 11 μM) with the library (1 μM of each compound) listed in Table 1. After incubation to promote the binding event, unbound drugs (filtrated solution) and bound drugs obtained from the denaturation of proteins were analyzed by DIOM-MS. First, the HSA-complex and unbound compounds in the library were easily separated by ultrafiltration (5,000 Da cutoff) The bound compounds were subsequently released by the addition of 60% methanol (200 μL) to denature the HSA. An additional washing step was also needed before the denaturation step to completely remove nonspecifically bound drugs from the protein and the Vivaspin filter in order to prevent potential false positive results. Finally, the resulting fraction, which contained the drugs that specifically bound to HSA, was reduced and concentrated to be easily detected by DIOM-MS. Although both positive and negative ionization modes are possible in DIOM-MS, the positive ion mode was selected for detection as it showed greater sensitivity[17].

All of the drugs listed in Table 1 were examined for binding to HSA. A control experiment without HSA was also performed to validate the proposed method. Figure 3a is the positive reflectron DIOM mass spectrum of the 11 candidates without HSA, with each peak labeled with its corresponding number from Table 1. Figure 3b shows the mass spectrum of compounds released from protein-drug complexes after incubation with HSA. Comparison of the mass spectra clearly confirmed that the binding compounds were 5, 6, 8, 9, 10 and 11, which agrees well with previous results in the literature[12,15,18-20]. Naproxen (no. 5) has the strongest primary association constant (Ka) of $1.6 \times 10^6\ M^{-1}$, whereas alprenolol (no. 8) has the weakest binding with Ka of $1.0 \times 10^3\ M^{-1}$ [8]. These binding constants indicate the minimum Ka needed for screening candidates from a library by DIOM-MS/ Ultrafiltration. If a drug has an association constant (Ka) of $1.0 \times 10^3\ M^{-1}$ or lower, it cannot be screened as a binding drug because of either a low signal intensity or a total loss of molecular ion intensity after binding with soluble HSA.

We demonstrated herein that this novel metho-

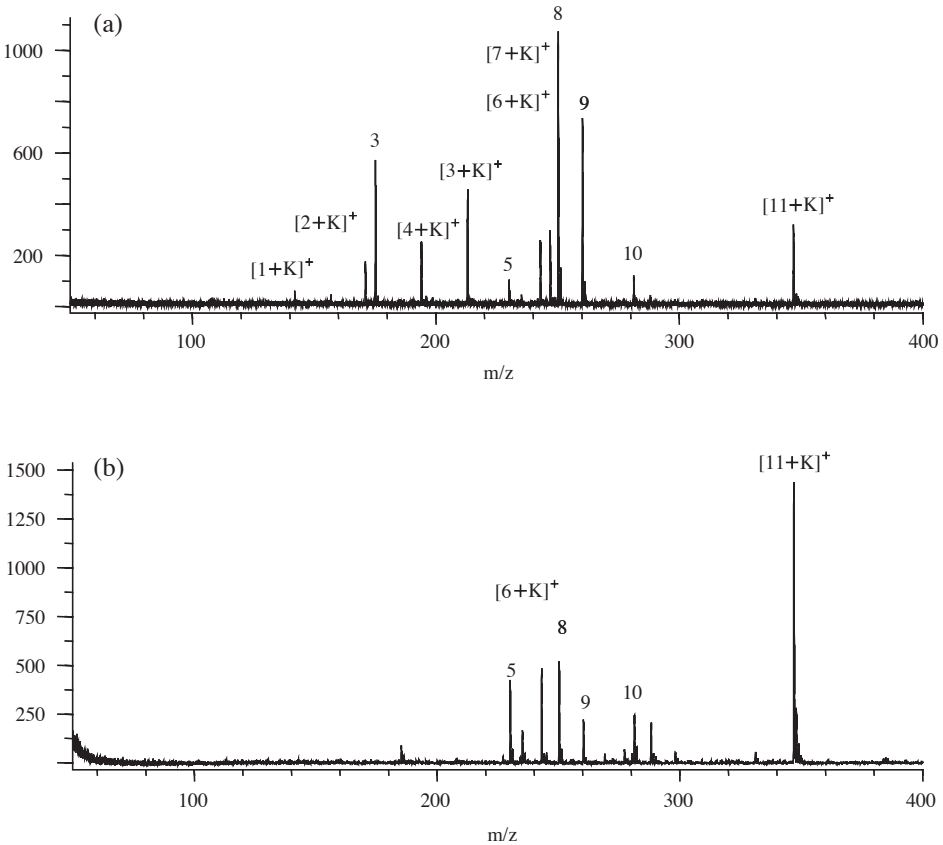

Figure 3. DIOM-MS spectra of binding drugs. (a) control experiment without HSA, (b) drugs released from HSA.

dology using DIOM-MS and ultrafiltration can be applied to drug screening. Because a mixture of 11 compounds was incubated with the target protein at the same time under native solution-based conditions, this method greatly reduced the time required for the separation procedure and increased the number of samples analyzed per experiment. In addition, compared with other approaches to alleviate matrix-related background peaks, the advantage of DIOM-MS is the relatively simple preparation of materials with ordered structure, which renders efficient performance without noise in the low mass range.

Conclusions

A novel approach shows that the combination of ultrafiltration with DIOM-MS can potentially be used as a drug screening tool for small molecules. This method has the potential for high throughput as it is simple, robust and rapid with greatly reduced separation and detection times. Unlike other affinity assays that use immobilized proteins, the data presented in this study resulted from real binding events under native solution-based conditions and showed relatively high sensitivity. Another strong point is the performance of DIOM-MS in the analysis. DIOM-MS provides clean mass spectra without any noise signals, which is an important issue in the analysis of small molecules by mass spectrometry. Our proposed matrix-free approach, DIOM-MS, is a simple but reproducible and powerful method to analyze low-mass molecules and has the potential for high throughput.

Therefore, further development of this method could provide a high throughput drug screening that can be used in preliminary target drug discovery.

Materials and Methods

Materials

Alanine, Gly-Asp, γ-aminobutyric acid, ornitine, arginine, atenolol, caffeine, naproxen, alprenolol, ketoprofen, imipramine, histidine, tryptophan, kynurenine, propranolol, warfarin, α-cyano-4-hydroxy cinnamic acid (CHCA), and peptides (bradkynin, d-Arg-bradkynin, melanocyte stimulating hormone) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). All chemicals used in this study were analytical or research grade. Deionized water was

obtained by a Milli-Q-Plus water system from Millipore (Waltham, MA, USA).

Synthesis of Mesoporous Silicates (Al-SBA-15)

In our previous study, we reported the optimized composition of mesoporous silicate, Al-SBA-15 ($SiO_2 : Al_2O_3$=25 : 1), which was prepared by hydrothermal template synthesis routes[17]. Briefly, a template solution was prepared by the addition of poly (ethylene oxide)-block-poly (propylene oxide)-block-poly (ethylene oxide) tri-block copolymer (BASF, Pluronic P123: $EO_{20}PO_{70}EO_{20}$) to HCl solution (1.6 M in H_2O). The template solution was stirred until homogenous. A silica source, tetraethyl orthosilicate (TEOS), was added to the template solution with vigorous stirring until clear. An aluminum source (i.e. aluminum chloride hexahydrate) was subsequently added to the clear TEOS and template solution. The first hydrothermal step (308 K, 24 h) and the second step (358 K, 24 h) were carried out in a mechanically stirred reactor equipped with a condenser and thermometer. Finally, the solid product was recovered by filtration and air-dried at 373 K overnight. The synthesized Al-SBA-15 was calcined in air at 823 K for 5 h using a muffle furnace. Figure 1(a) shows the SEM image of the prepared mesoporous silicate, Al-SBA-15 ($SiO_2 : Al_2O_3$=25 : 1), with its large surface area (959 m^2/g), nano-scaled pore size (average 5.9 nm), and highly ordered pore shape (i.e., hexagonal array)[17].

Drug Binding Assay and Ultrafiltration

HSA (11 μM) in 25 mM ammonium bicarbonate buffer at pH 7.5 was prepared, and drug ligands were diluted to 1 μM with the same buffer solution. The drug library used for this study was labeled and is listed in Table 1. Incubation mixture was prepared by incubation of the drug library with excess HSA (11 μM) for 15 min at 37°C. The samples were then subjected to ultrafiltration. Vivaspin concentrators with 5,000 MW cutoff (Satorius, USA) were used to remove unbound library drugs library following the incubation; the Vivaspin with a cutoff of 5,000 MW can separate high MW compounds from low MW compounds. Separation was carried out in a table-top microcentrifuge (Hanil, Korea). First, the Vivaspin was equilibrated with ammonium carbonate buffer for 1 min at 3,000 rpm. The incubation mixture containing free drug, free HSA, and HSA-drug complex was loaded and centrifuged at 3,000 rpm for 3 min. Small molecular weight unbound drugs rapidly passed through. The remnant then contained free HSA and HSA-drug complex, including selected candidates with high binding affinity. To release bound drugs from HSA, 60% methanol was added to the remnant to denature the HSA protein, so that denatured HSA would easily release the bound drugs. The removed drugs were collected and sequentially analyzed by DIOM-MS. These procedures are depicted in Figure 2.

Mass Spectrometry

The overall mass spectrometric analysis was performed by time-of-flight mass spectrometry (Biflex IV, Bruker, Germany). A 337-nm nitrogen laser was used for desorption/ ionization. The ions were accelerated under delayed extraction with an accelerating voltage of 20 kV in the positive or negative ionization mode with an average of 100 shots. The analytical range of laser power was adjusted to obtain good resolution and signal-to-noise ratio. Mass calibration was performed with external standards including a mixture of alanine (Mw: 89.0941), β-Gly-Asp (Mw: 190.0589), bradkynin 1-5 (Mw: 572.3070) and bradkynin 2-9 (Mw: 903.4603).

To demonstrate the DIOM-MS method, Al-SBA-15 (1 mg/mL, 0.25 μL) from the homogenized particle suspension in volatile pure methanol solvent was first deposited on a stainless steel plate (Figure 1b). Analytes were subsequently added and air-dried. All measurements were performed in triplicate, and the average values of the measurements were acquired from X-TOF and Biotools 2.0 program (Bruker, Germany).

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