An ultra high throughput, double combinatorial screening method of peptide–metal binding

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An effective ultra-high throughput, double combinatorial method of screening potential selective ligands based upon oligopeptides is described. This rapid screening of bead-based libraries by Micro X-ray Fluorescence (MXRF) was used to identify selective chelating agents for metals that may be found in radioactive dispersive devices (RDDs). The method has proven to be a powerful tool to rapidly and quantitatively screen metal–ligand interactions. It is a tag-free, sensitive technique, which in a combinatorial approach with peptide libraries (*e.g.* varying charge, length, hydrophobicity, ligand elements *etc.*), provides a rapid and quantitative means for identifying metal–ligand interactions.

Introduction

High throughput screening (HTS) of combinatorial libraries is a tool that is widely used in the drug industry because hundreds and thousands of compounds can be routinely synthesized and analyzed in a short period of time. Better, cheaper, and more effective drugs have been discovered through this approach. However, in the inorganic chemistry community, the target-design process is still the main tool to predict the physical and structural properties of new ligands. In many cases it is not practical to employ specific ligand design and synthesis. In complex systems, specifically environmental applications, coordination events may be highly competitive between large numbers of metals. The identification of a selective ligand is often a time-consuming process which tasks the skill of the synthetic chemist. In applications where ligands are needed for environmental cleanup a linear approach may not be practical. With the continuing threat to our environment by toxic metals and in particular the potentials for new radiological threats the need for selective and specific ligand discovery is a major challenge. A wide array of analytical methods based on mass spectrometry,[1–3] infrared thermography,[4] Fourier transform infrared spectroscopy,[5,6] near-infrared spectroscopy[7,8] and laser-induced fluorescence imaging[9] have all been adapted for routine characterization of combinatorial libraries. However, most of these methods focus on molecular features, which can be detected *via* spectroscopic based techniques. X-Ray detection techniques uniquely and specifically target elemental features.

Several X-ray based methods have been demonstrated in applications highlighting the capability for both qualitative and quantitative analyses of the elemental signature of given molecular interactions. Micro X-ray fluorescence (MXRF) in particular has unique capabilities ideally suited for high throughput screening. MXRF offers spatially resolved elemental characterization using either single point or elemental imaging capabilities. These unique features allow MXRF to be applied to a wide variety of high throughput screening applications including catalysts, fuel cells, pharmaceuticals and materials. As long as there is an element, which can be excited and detected (typically any element with $Z > 10$) *via* X-ray fluorescence within the combinatorial experiment, MXRF can be used for high throughput screening of the lead material.

In the past we have employed MXRF to identify ligands for catalytic applications,[10] but the issue of developing specific ligands for metals in complex mixtures poses a much greater challenge. Herein we report the development of the MXRF technique to incorporate a double combinatorial approach, *i.e.* screening multiple metals against multiple ligands, essentially providing an ultra high throughput screening of specific peptide-based metal binding agents. We have identified selective oligopeptide chelators for radioactive metal ions, which may be found as contaminants after an RDD or dirty bomb incident.

The foundation for MXRF in HTS has previously been described by Miller *et al.*[10] In brief, the X-ray beam excites the elements present in the target. The excited elements emit X-rays that are characteristic to the elemental composition of the sample. The use of a polycapillary X-ray optic enables the detection of nanogram levels of the target element.

MXRF is unique when compared with conventional high throughput detection techniques such as fluorescence, ultraviolet spectrometry and other methods used in molecular recognition. The distinctive feature of MXRF is the detection of the native elemental fluorescence signature of the combinatorial reaction species. Techniques such as molecular fluorescence require the molecules of interest to be naturally fluorescent or the molecules to contain a fluorescent tag. While metals have the advantage of often generating or reducing

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fluorescence or colour upon coordination this is not a universal property for all ligand–metal combinations. It is therefore often necessary to attach reporter groups to ligands to identify interactions. This restriction limits the range of reactants, simply due to the time it takes to prepare the labeled materials. In addition added tags or labels are often bulky and can adversely interfere, both sterically and chemically, with the specific molecular recognition chemistry. Furthermore, fluorescent tags can undergo rapid photobleaching when measurements are performed with high spatial resolution similar to MXRF. MXRF avoids these problems since the method does not require added tags, but simply an intrinsic detectable heteroatom, with atomic numbers greater than sodium, to be present for analysis. MXRF also provides both qualitative and quantitative measures of the effective reactions as detected by the elemental intensities. This allows one to not only identify the lead hits, but to quantitatively evaluate the quality of the hits. The quantitative evaluation provides a means to rank the hits and pursue the most promising ones in a more effective and productive follow up on the leads.

The straightforward approach for MXRF high throughput screening involves the use of a spatially separated combinatorial library and either single point spectrum acquisition or elemental or spectral mapping. In the elemental mapping mode, the combinatorial library is rastered by the focused X-ray beam and the emitted elemental intensities for preselected elements are detected at each position. The dwell time, area scanned, number of pixels and step size all determine the overall resolution of the elemental map and ultimately the sensitivity. Typical applications will scan an area about 16 × 15 mm at a time. In an optimized mode and with registered substrates, an array format for data acquisition can be instituted so only the beads are analyzed using single point regions of interest or full spectrum acquisition. This significantly reduces the scan time to only points containing materials of interest. The full spectrum approach provides correlating elements as well as detecting unexpected results. Although this generates significantly larger data files, it is the most comprehensive for information content.

By subjecting the bead-based library to a mixed metal solution, which not only includes the metal of interest but other potential interfering metals, a double combinatorial, ultra high throughput technique is developed. Using the method presented herein 25 700 sequences can be analyzed per day. In this study we were limited to a library with only 8000 sequences. If we consider that we simultaneously study the specific reaction of each metal, not just the metal of interest then we can say that the number of experiments in a day is M × L where M is the number of metals and L is the number of ligands. In this study the binding efficiency of various oligopeptide libraries with the metal of interest (Co^{2+}) in the presence of ten potential interfering metal ions were analyzed. Consequently the number of experiments performed per day is in the range of 280 000.[11] It is therefore, now possible to screen a library of chelators A against a library of metals B, or any compound containing an element $Z > 10$, in a rapid and very selective manner.

Results and discussion

The ultra-high throughput, double combinatorial technique allowed the screening of 8500 chelating agents against eleven target metals (Co^{2+} plus ten potentially interfering metal ions) in pH conditions that we would expect to encounter in an urban enviroment. On a lab scale this is equivalent to a daily rate of approximately 280 000 bench top experiments. The HTS step rapidly identifies which libraries are worth screening more carefully thereby-eliminating hours of unnecessary screening. The elimination of the synthetic step of tagging the peptides avoids not only the added expense but also, and more importantly, the reporting of false positives or negatives.

Of the libraries screened, no Co binding agents were observed with the 3-mer petide library and 15 hits were identified with the 4-mer peptide library. Fig. 1 illustrates a typical area scan of the 4-mer peptide library, the identification of a Co binder and the validation with a point spectrum indicating a high intensity for cobalt. These hits were located and the beads removed from the Tacky Dot™ slide. Replacing the bead in the MXRF instrument and checking the cobalt intensity confirmed the presence of Co on these beads. The X-ray intensities for cobalt (emission line energy = 6.92 keV) and other elements (Fig. 2) were recorded for each bead. These X-ray intensities allowed us to rank the hits in order of binding efficiency. The four most efficient binders were sequenced (Table 2) and the experiment repeated with a batch of the known sequence. Although the presence of histidine in the sequence is unsurprising as it is a common ligand for transition metals, the presence of amino acids such as proline, leucine

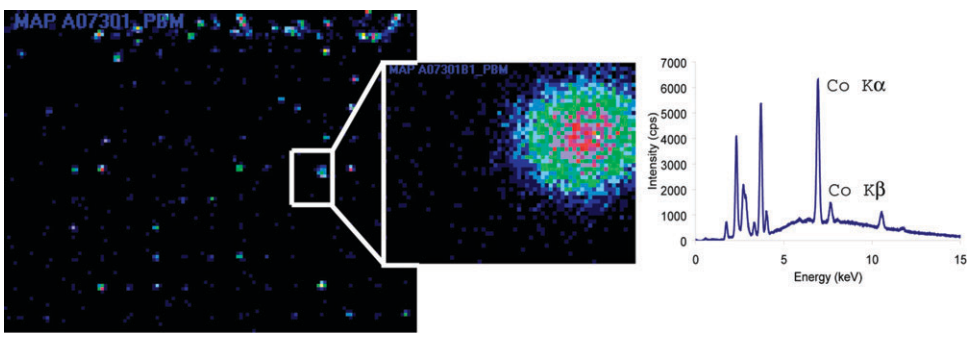

Fig. 1 A cobalt elemental spectral image of a peptide bead library on Tacky Dot™ slides reacted with cobalt in the presence of potentially interfering metals. A hit is identified and a spectral image of the bead localizes the intensity of Co, which is verified by a point spectrum.

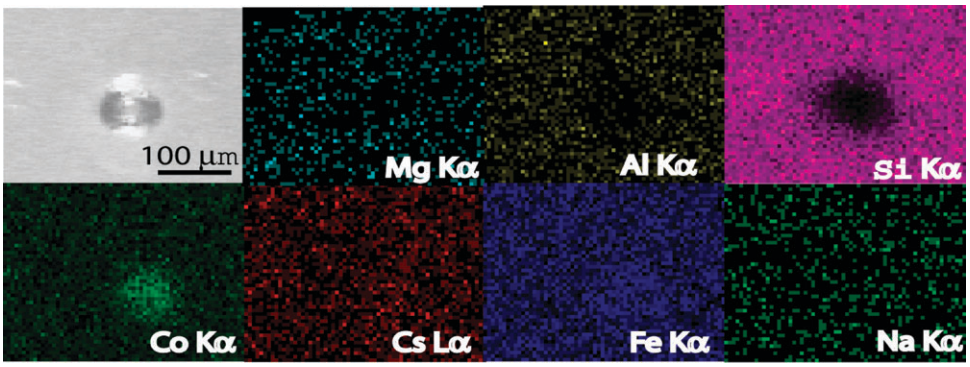

Fig. 2 Elemental mapping of a single peptide bead bound to cobalt. Top row: optical image of bead, Mg mapping, Al mapping, Si mapping (shows up negative due to the Si present in the glass bead). Bottom row: Co hit identified; Cs mapping, Fe mapping and Na mapping all prove to be negative.

and valine were not intuitively expected. It is clear that sequences that may otherwise have been ignored as potential ligands have been identified by this method. Fig. 3 shows a typical scan of an array with the identified Co binding peptide sequence. The presence of Co is indicated by the intensity of the cobalt signal. It should be noted that as a result there is some variation in even these samples, which is likely due to the purity of the peptide on the beads (the manufacturer quotes a maximum of 70% purity due to the nature of the peptide synthesis). The varying intensity of the cobalt is likely due to the lack in homogeneity of peptide coverage on the bead. Other issues arise in that it is possible that the nature of binding on the bead is completely different than the free ligand in solution.

Experimental

All reagents were commercially available and used without further purification. Unbiased libraries of bead-based oligopeptides with protecting groups removed were used, American Peptide Inc. (Sunnyvale, CA). Tacky Dot™ slides, SPI Supplies (Westchester, PA) were the substrate used for the immobilization of the beads. A standard metal solution was made of the following metals Co, Cs, Ca, Al, Fe, Ir, Sr, Mn, Mg, Na, and K (7 mM of each), High Purity Standards (Charleston, NC). pH adjustments were made with 1 M NaOH, Acros (Morris Plains, NJ). An in-house produced polyvinylalcohol (PVA)-based polymer was used.

X-Ray excitation and detection were performed using an EDAX Eagle II micro X-ray fluorescence system with a Rh target excitation source and a SiLi detector (EDAX, Mahwah, NJ) having a 50 µm nominal X-ray spot size. X-ray tube operating conditions were maintained at 35 kV and 400 µA. Each Tacky Dot™ slide (2.5 × 5.5 cm) was divided into six areas (1 cm^2 approx.), allowing a 256 × 200 pixel array with a 100 ms dwell time. Beads identified as "hits" were subsequently analyzed individually by collecting a single point spectrum. The single point spectra were acquired with an integration time of 100 live seconds on the detector. Table 1 lists the element emission lines and their energies that were monitored in this study. Beads identified as hits were removed by hand with the aid of a Leica Micro Star IV stereomicroscope (Leica Microsystems, Bannockburn, IL).

Edman degradation experiments were carried out by MTC Protein Sequencing & Analysis Services (Tucson, AZ) on a 477A protein sequencer (Applied Biosystems, Foster City, CA).

20 mg of a Wang resin-bound 3-mer oligopeptide combinatorial library was immersed in a polymeric-based mixed metal solution (1.5 mL, 7 mM, pH 10) and mixed for 12 h. After this time the beads were filtered by gravitational filtration, washed three times with 15 mL of deionized water and air-dried for 12 h. The dried beads were then deposited between two sheets of polypropylene, 4 µm thick (Spex Certiprep, Metuchen, NJ), which was fixed to a sample cup. The sample was split into two areas for analysis. In this way, batch analysis was carried out, providing a rapid initial screening of the library. An area was scanned overnight and if hits were observed, the beads were subsequently immobilized onto a Tacky Dot™ slide(s) and reanalyzed by MXRF. Emission lines were monitored for all

Table 1 The top four sequences that preferentially coordinate cobalt

Sequence[a]	Intensity of Co K_α/cps
IHHS	194
PLDG	152
PVLG	118
KRHH	106

[a] I: isoleucine, H: histidine, S: serine, P: proline, L: leucine, D: aspartic acid, G: glycine, K: lysine, R: arginine, V: valine.

Table 2 Elemental emission lines monitored

Element	Emission line	Energy/keV
Co	K-$L_{2,3}$ (Kα)	6.92
Ca	K-$L_{2,3}$ (Kα)	3.69
Cs	L-$M_{4,5}$ (Lα)	4.29
Al	K-$L_{2,3}$ (Kα)	1.49
Mn	K-$L_{2,3}$ (Kα)	5.89
Sr	K-$L_{2,3}$ (Kα)	14.14
Na	K-$L_{2,3}$ (Kα)	1.04
K	K-$L_{2,3}$ (Kα)	3.31
Mg	K-$L_{2,3}$ (Kα)	1.25
Ir	L-$M_{4,5}$ (Lα)	9.14
Fe	K-$L_{2,3}$ (Kα)	6.40

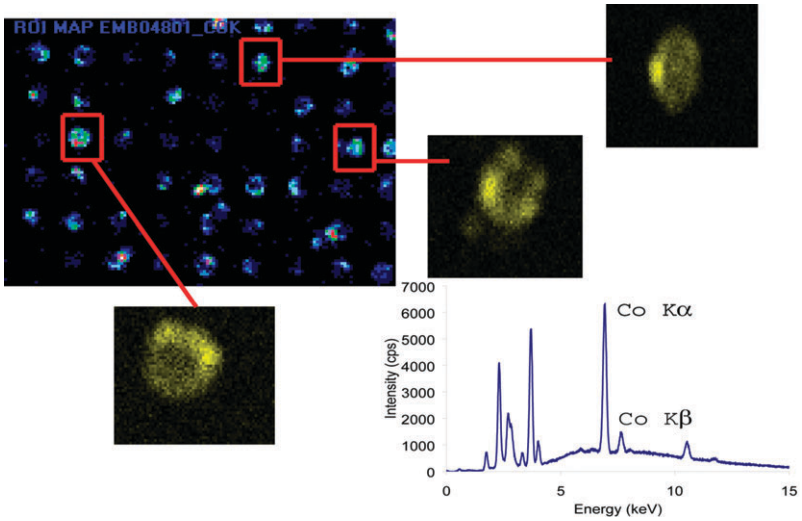

Fig. 3 An MXRF study to confirm coordination of Co to beads with the peptide sequence IHHS. The Tacky Dot™ array highlights the binding events. Spectral images of the beads show the inhomogenity of the peptide on the bead. Cobalt intensities for each binding event are spectroscopically compared.

elements listed in Table 1 but more specifically for Co (K-$L_{2,3}$ (Kα)). This was repeated using 4-mer and 5-mer combinatorial libraries enabling the screening of a total of approximately 8500 potential binding agents. Hits were identified, isolated and their amino acid sequence determined by Edman degradation. The sequences were correlated to their respective Co intensities.

This paper demonstrates the analytical or HTS capability of MXRF for combinatorial screening. It is meant only to show the capabilities of MXRF and is not meant as an exhaustive study of the molecular recognition systems presented.

Conclusions

Micro X-ray fluorescence is an extremely useful tool in the screening of ligands. Moreover, this study demonstrates the enormous potential that MXRF possesses as an ultra high throughput-screening method. Here the double combinatorial technique has been developed to identify lead binding agent for a specific metal in a particular application by mimicking the expected conditions including potential interferences at the experimental stage. The results also highlight the importance of an unbiased library, providing new, counter-intuitive sequences for the binding of Co. Although peptides are the chelating agents of choice for this study, the method can be applied to the screening of a variety of chelating compounds.[12] It is a non-destructive technique with a nanomole detection limit.

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