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Magnetic Extraction, Detection, and Isotope Analysis of Metal Ions Using Surface Modified Microspheres for Lab-on-a-Chip Applications

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Liquid–liquid extraction and ion-exchange chromatography are efficient methods for sequestration of metal ions, but these methods are poorly suitable for microfluidic miniaturization. Here we examine several methods for rapid extraction, fluorometric detection, and the subsequent isotope analysis of lanthanide ions sequestered on surface modified magnetic microspheres as a versatile platform for chemical manipulation. The assays involve immobilization of a mixed-ligand complex of luminescent lanthanide ions at the surface of polystyrene microspheres. Using the ion-exchange properties of these microspheres, this scheme can be extended to the detection of nonluminescent ions.

Keywords magnetic separations; ion-exchange chromatography; microfluidics; fluorometry; isotope analysis; antenna effect

INTRODUCTION

Separation and detection of trace amounts of metal ions, including fission products and other radionuclides of interest for nuclear forensics, frequently involve preconcentration of these ions by using liquid–liquid extraction or ion exchange chromatography (1,2). Adapting these techniques to microfluidic devices is challenging, whereas the miniaturization and speeding up of the complex "wet" analytical procedures is highly desirable. The question we address in the present paper is: how to adapt these methods to the constraints imposed by the design of portable microfluidic devices? What can we replace mixing of aqueous and liquid organic phases or stationary polymer phases with? How do we perform microfluidic sequestrations in a manner that is maximally conducive for the subsequent detection and isotope analyses?

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One possible approach is using complementary techniques for manipulation and separation of the ions, such as electrophoresis (3). However, the latter method is poorly suited for many samples due to the high conductivity of acidic solutions necessary to stabilize the ions. Moreover, there is a wealth of the already established separation chemistry that would benefit the elemental and isotope analyses in a lab-on-a-chip. It has been realized that magnetic microspheres (MMs), which are relatively easy to manipulate in microfluidic channels, can potentially replace these liquid and solid phases (4,5). The first examples of using the microspheres as the solid support for immunological and DNA hybridization assays in microfluidic setting began to appear in the literature (6). These developments encouraged us to seek the use of the MMs for manipulation of metal ions and develop MM-based assays for sensitive detection and isotope analyses of trace amounts (<1 ppb) of ions immobilized on these MMs. These assays serve as the basis for operation of a portable microfluidic device for nuclear forensics applications that is being developed in our laboratories.

While we are not first to use the MMs for metal ion separations (7–15), our approach is different from these previous studies. The MMs have been modified either by impregnation of the MMs with neutral ligands (7–11) or by covalent binding of the ligands (12–15). The former approach was successful from the extraction chemistry standpoint, (7–15) but the resulting material was aggregated, which makes it poorly suitable for microfluidic applications, whereas the second approach (12–15) focused on sequestration with little regard to the needs of the subsequent analyses. Our approach, by contrast, is constrained by both of these requirements: the microspheres are fully suspendable for ease of manipulation in a microfluidic device and the complexation of metal ions facilitates the subsequent detection and the isotope analyses.

The principle of the assay (Fig. 1a) is similar to the well-known DELFIA immunoassay, (16–21) in which Eu^{3+} forms a luminescent complex (LC), such as Eu^{III} L_2A_3,

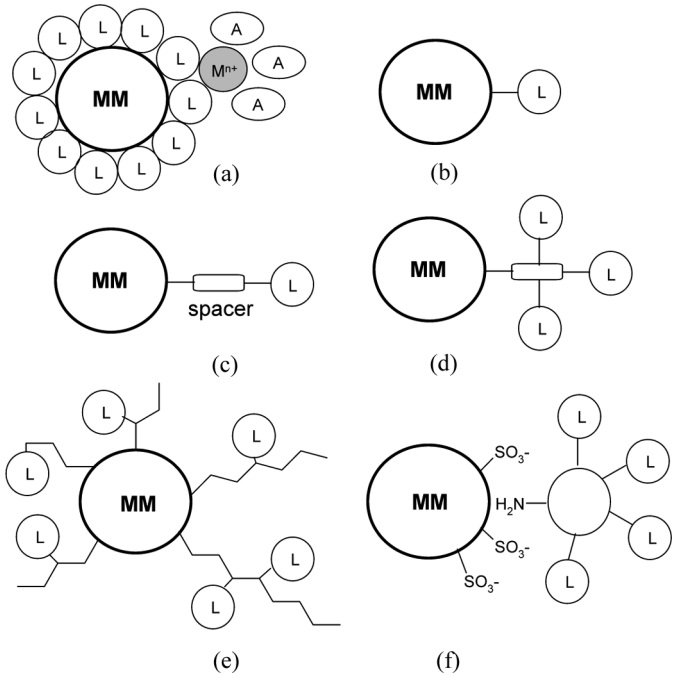


FIG. 1. Schemes for ligand attachment to the MMs: (a) "physisorption" approach, (b) direct attachment to carboxyl and amino groups at the MM surface, covalent attachment to tethered, (c) mono- and (d) poly-functional spacers, including, (e) polymers and (d) dendrimers. Trace (i) is the background, trace (ii) is the luminescence developed in the presence of the MMs and trace (iii) is the signal observed after a magnetization/resuspention cycle.

where *L* is a neutral ligand, such as tri-*n*-octylphospine oxide (TOPO) and *A* is an antenna ligand, such as the base of 2-thenoyltrifluoroacetone (TTA, $pK_a \approx 6.32$). The ligands are extracted into and the LC is formed inside the core of a nonionic micelle, such as Triton X-100. This detergent has an aromatic "head" and hydrophilic poly-ethylene glycol (PEG) "tail." The hydrophobicity of the aromatic core precludes rapid quenching of the Eu^{III} luminescence by water ligands, as the excitation energy of the relatively long-lived emissive state of the LC is vibronically dissipated by these ligands (22–24). The antenna ligands absorb the excitation light and the energy of the triplet state is transferred to Eu^{III} that emits at 620 nm via $^7F_2 \leftarrow {}^5D_0$ transition, with the emission lifetime >0.5 ms. This extremely long luminescence allows background free detection of as little as 1 ppt of Eu^{3+} by means of time-resolved laser-induced fluorescence spectroscopy (TRLF) (19). The fluorescence of the unbound ligands and the luminescence of Eu^{III} ions that are not fully coordinated by these ligands is short-lived, and so it evades TRLF detection in the pre-set time window. This makes TRLF a sensitive, background free technique for the detection of the luminescent ions, such as Eu^{III}, Tb^{III}, and Er^{III}.

In DELFIA immunoassay (16–21), Eu^{III} is bound to ethylenediamine tetraacetic acid (EDTA) conjugated to a protein of interest. Following the assay, the luminescence is developed by addition of TTA and tri-*n*-octylphosphine oxide (TOPO) in Triton X-100 and detected using TRLF. This chemistry, and the formation of mixed-ligand complexes in general, has attractive features for magnetic sequestration. A capture ligand is attached directly to the MM, whereas the second ligand can serve as a reporter, either by the antenna effect (for luminescent ions) or by fluorescent dye tagging. Following the sequestration and the fluorometric detection, the ions can be stripped off the MM for subsequent mass spectrometry (MS) analysis; alternatively, the ions can be detected using laser desorption ionization MS on the MMs.

Below we examine several strategies by which this approach can be used for the MMs. The two classes of neutral ligands that we examined were phosphine oxides (such as TOPO) and diglycolamides. Both of these ligands are known to form stable mixed-ligand complexes with TTA (16–25). Figure 1 illustrates the general approach: the ligands were attached to the MMs by

(a) physisorption, (b) covalent attachment to carboxylic and amino groups at the surface or via, (c) mono- and (d) poly-functional spacer molecules, including (e) polymers and (f) polyamidomine (PAMAM) dendrimers.

EXPERIMENTAL

Magnetic Microspheres

We examined a number of commercially available MMs, including cellulose, silica, and polystyrene microspheres obtained from different suppliers (including Spherotech (Lake Forest, Illinois), BioClone (San Diego, California), Pierce Biotechnology (Rockford, Illinois), Cortex Biochem (Madison, Wisconsin), and Micromod Partikeltechnologie (Rostock-Warnemuende, Germany)). The typical morphology of these microspheres can be obtained from the specifications provided by the manufacturers (e.g., reference (26)); surface modification described in this section does not change this basic morphology. All but one brand of these MMs were shown to be efficient luminescence quenchers for the lanthanide ions due to the presence of dispersed Fe^{3+} ions near the surface, to which the excitation energy is transferred. Spherotech (Lake Forest, Illinois) supplies polystyrene microspheres that have 1 μm thick protective overcoat of the polymer which isolates these ferric ions (26) and only such MMs were shown to support long-lived luminescence from immobilized lanthanide ions. These MMs are polymerized using $K_2S_2O_8$, and so the microspheres have sulfonate groups at their surface that stabilize the MMs in an aqueous solution. These "smooth" MMs are provided as a 2.5 wt% liquid suspension. Carboxylated and aminated "smooth" MMs were obtained from the same supplier. The properties of these MMs are given in Table 1. The MMs were stabilized using 0.1 wt% decanoyl-N-methylglucamide (MEGA10).

TABLE 1
Properties of "smooth" polystyrene MMs [a]) (Spherotech)

MM type	D[b], μm	A[c], × 10^7 nm^2	Surface group	n_g[d], per nm^2	N_g[e], × 10^8	concentration of MMs × 10^9 cm^{-3}[a]	concentration of MMs, pM[a]	volume concentration of groups, μeq/L[a]
PMS-20	2.5	1.96	–	–	–	2.8	4.66	–
CMS-30	4.67	3.4	CO_2^-	4.2	1.4	1.24	2.06	293
AMS-40	3.28	6.9	NH_2	1.7	1.8	0.42	0.7	83

[a]For a 2.5 wt% stock aqueous solution.
[b]Median diameter.
[c]Surface area per MM.
[d]Surface density of groups.
[e]Total number of groups per MM.

This nonionic detergent was chosen for its high critical micellization concentration (cmc) of 0.25 wt%. Thus, it was possible to cover the MMs with this detergent (preventing the aggregation of the microspheres) without forming micelles in the solution. The latter readily extract the LC into their hydrophobic interior and thereby enhance the luminescence of the complex.

Reagents

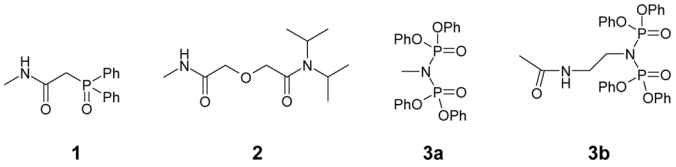
Unless specified otherwise, all chemicals and solvents were obtained from Sigma-Aldrich (Milwaukee, Wisconsin) and used in their purest available form. The standard solutions of Eu^{3+} containing 10^{-4} M HNO_3 were prepared from europium(III) nitrate pentahydrate. This pH has been chosen to minimize Eu^{III} hydrolysis ($pK^* \approx 6$) (27).

Time-Resolved Fluorescence Measurements

Liquid samples (0.3–1 mL) were placed in a 1 cm × 1 cm polystyrene cuvette. No deaeration of the samples was necessary. The sample was photoexcited using a 6 ns, 1 mJ pulse of 355 nm light from a Nd:YAG laser (Quantel USA, New York, New York). The laser beam had the diameter of 6 mm. The emitted light was collected at 90° and passed through a narrow band (40 nm fwhm) interference filter with the transmission maximum at 620 nm. The signal was sampled using a fast photomultiplier (PMT) and terminated into 4 kΩ load at the model TDS 360 digitizing oscilloscope (Tektronix, Beaverton, Oregon). The PMT was operated at 0.3–1.2 kV, depending on the emission yield. For weak emission signals, the PMT output was amplified. Ten to 200 kinetic traces were sampled and averaged at 1–2 Hz. The typical kinetics are shown in Fig. 2. In the first 10 μs after the laser photoexcitation, the signal is dominated by the emission and scattered laser light from the MMs and the fluorescence from the organic molecules. The kinetic traces for delay time $t > 100$ μs were fit exponentially and extrapolated to the time origin. The amplitude of the extrapolated signal was used to quantify the luminescence yield, and the exponent of the least-squares fit provided the estimate for the lifetime of the luminescence.

Neutral Ligands for Surface Modification of the MMs

The ligands were covalently attached to the MMs and/or the spacers using amide conjugation protocol, and so the precursors of these ligands were either carboxyl- or amino-terminated. The syntheses of these ligands are given below:



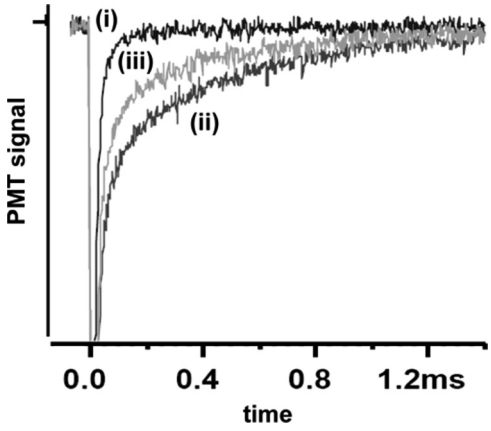


FIG. 2. TRLF kinetics obtained in an assay that involved AMS-40 microspheres modified with **4**. The concentration of Eu^{3+} is 120 pM (20 ppt) and the concentration of the MMs is 1.8×10^7 cm^{-3}. The 620 nm luminescence was developed by addition of 0.25 mM CMPO in 0.1% Triton X-100. The dotted line is the background, the solid line is the luminescence developed in the presence of the MMs and the dashed line the signal observed after a magnetization/resuspension cycle. The vertical axis gives PMT signal.

(Diphenylphosphoryl) acetic acid. The synthesis was based on the method described by Böhmer and co-workers (28). 4.9 g (22 mmol) of ethyl ester of diphenylphosphinous acid was placed in a dropping funnel and added over 10 min to 4.2 g of ethyl bromoacetate. After the evolution of ethyl bromide, the mixture was stirred at 70°C for 1 hr. The mixture was cooled and then 4.5 g of NaOH in 50 mL of 1:1 water/methanol was added, and the mixture was stirred at 25°C for 12 hr. The methanol was evaporated, water added, and the excess reagents were extracted into CH_2Cl_2. Hydrochloric acid (0.1 M) was added to the aqueous layer and the product was extracted into CH_2Cl_2. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to dryness. No further purification of 1 was needed, and the product was 99% pure by high performance liquid chromatography (HPLC) and ^{31}P and ^{1}H nuclear magnetic resonance (NMR). The product (10 mmol) was dissolved in dimethylsulfoxide (DMSO) and used in the subsequent syntheses, preactivated by N-succinimide (NHS).

N,N′-Di(isopropyl)glycolamide. The synthesis was adapted from refs. (29–31). 2.79 g (27.6 mmol) of glycolyl anhydride was dissolved in 30 mL CH_2Cl_2 and 3.2 g of neat di-isopropylamine was added. As the mixture began to "boil," 30 mL CH_2Cl_2 was added and the mixture was stirred for 1 hr, and then 1 mL of triethylamine was added and the mixture was stirred for another 12 hr at 20°C. The mixture was evaporated to dryness, the oil was dissolved in CH_2Cl_2, washed with water, dried over Na_2SO_4 and used as is. The net yield was 3.8 g of the oil, and the product **2** was 95% pure by HLPC.

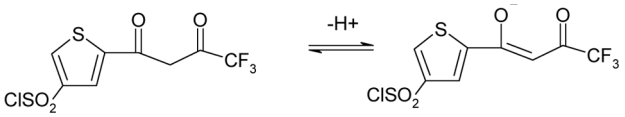
Di(diphenylphosphoric)imide. One to two mL's of 2.5 wt% of AMS-40 microspheres were rinsed with water and then washed six times with anhydrous dimethylformamide (DMF), by repeated magnetic separation and suspension. 20 mL DMF was added and then 200 µL diphenyl phosphochloride (or diphenyl phosphinic chloride) and 200 µL pyridine. The mixture was stirred for 24 hr at room temperature. The MMs are magnetically separated, washed ten times with 15 mL methanol, and then twice sonicated for 1 min in methanol. The MMs are separated magnetically, washed three times with 0.1% MEGA10.

Di(diphenylphosphoric)(2-aminoethyl)imide. 1 g of N-BOC-ethylenediamine (6.25 mmol) was dissolved in 10 mL CH_2Cl_2. 3.93 g of diphenyl phosphochloride in 70 mL CH_2Cl_2 with 1 mL Et_3N was added over 30 min. The mixture was refluxed for 10 hr and then evaporated to dryness. The oil was dissolved in CH_2Cl_2 and washed with brine, then with 1 M NaOH, and then again with brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. 10 mL of 1:2 mixture of CF_3CO_2H and CH_2Cl_2 was added to the oil to remove BOC protection. After 4 hr stirring at room temperature, the solution was neutralized by addition of 1 M KOH, and the organic layer was washed by brine and evaporated to dryness. The white crystals were twice recrystallized from acetone to give 1 g of HPLC pure product. The structure of 3b was confirmed by ^{31}P and ^{1}H NMR. The monosubstituted product was not present. The amino terminus of this molecule was used for amide conjugation.

Activated 2-Thenoyltrifluoroacetone Ligands for Surface Modification of the Microspheres

TTA was functionalized by reacting TTA with chlorosulfuric acid in CH_2Cl_2 for 75 hr followed by water quenching, drying of the organic layer with anhydrous Na_2SO_4, evaporation to dryness, and recrystallization of the product from cold *n*-hexane (32,33):

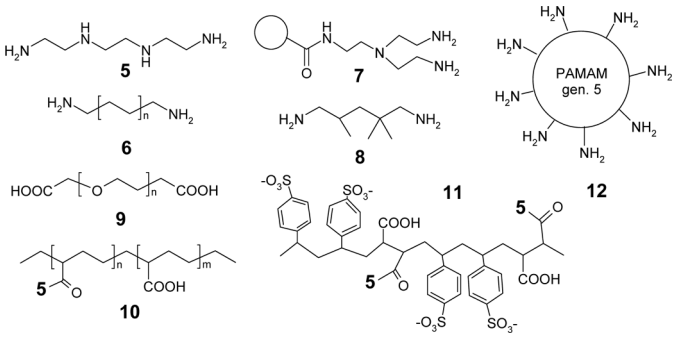
4



No other chlorosulfonation products were observed by ^{1}H NMR in the purified **4**, which was obtained with 70% yield. This derivative can be reacted with amino groups of the MMs or the spacers in dry DMF using Et_3N as a catalyst. Alternatively, **4** was first conjugated to **5** and then the product was amide conjugated to the carboxyl groups of the MMs or the spacers.

Spacers

The chemical structures of the spacers are given below:



These spacers served to link the ligands to the MMs (Figs. 1b–f). For most of these spacers, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) amide conjugation protocol has been used (34), in which the activated carboxyl groups were reacted with the amine in a pH = 5.5 phosphate saline buffer (PBS). The excess of the amine was added and the reaction mixture was agitated for 5–12 hr using an orbital shaker. DMSO was added to improve the solubility of the ligands and the spacers and prevent

the aggregation of the MMs during the conjugation. Following the reaction, the MMs were magnetically separated and reacted with 10 mM tris(hydroxymethyl) aminomethane (TRIS) for 5 min. The MMs were then washed with methanol (5–10 cycles of magnetization and suspension, with periodic 1 min sonication), and finally washed by the buffer or detergent solutions. Alternatively, the carboxylic groups were activated by EDC/NHS in DMSO or 3:1 v/v DMF/DMSO and reacted with the aminated (modified or unmodified) MMs. The latter were washed with dry DMF prior to the conjugation multiple times. For water-soluble polymers **10** and **11**, the attachment of the hydrophobic groups **1–3** resulted in the loss of solubility. To counteract this tendency, the carboxylic terminated ligands were first conjugated with **5** using EDC/NHS protocol in DMF, and these conjugates were then further conjugated with the carboxyl groups of the polymers (10–20% replacement) in DMSO/buffer solution using the standard EDC protocol. 3b was directly conjugated to the carboxyl groups. The ligand-tagged polymers and dendrimers were dialyzed in 5–10% NaCl and then conjugated (Fig. 1e), using their unreacted carboxyl- and amino-groups, to the MMs. For amino-terminated, generation-5 (128 amino groups) PAMAM dendrimer **12**, ion attraction between the amino groups of the dendrimer and the sulfonate groups of the MMs was sufficient for full coverage of the MM surface (Fig. 1f). Alternatively, the MM were first conjugated to the polymers/dendrimers and the conjugate was reacted with the excess of the functionalized ligand. The MMs were repeatedly washed by methanol and stored in 0.1% MEGA10 or Triton X-100 solutions.

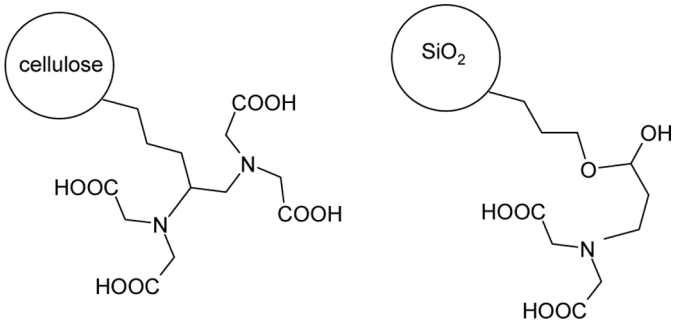
To estimate the sorption of the macromolecules at the MM surface, these were additionally tagged using NHS activated 5(6)-carboxy-X-rhodamine dye (RB). This dye was photoexcited using 532 nm, 5 mW light from a Nd:YAG diode pumped laser (B&W Tek, Newark, Delaware). The 532 nm light is not absorbed by the TTA. The excitation light was chopped at 200 Hz and the 600 nm emission was collected at 90° using a monochromator and a PMT. The PMT signal was demodulated using a SR810 lock-in amplifier (Stanford Research Systems, Sunnyvale, California).

In the attempt to grow a thicker coat that would more closely mimic a layer of the organic solvent in impregnated ion-exchange resins (1,2), polymer chains were grown directly on the MMs, by the amide conjugation. One of these designs involved ε-aminocaproic acid; another involved the monomer synthesized by reacting a 6 kDa diamino-PEG with glycolyl aldehyde (that integrates the diglycolamide functionality into the polymer). The resulting "coated" MMs were suspendable and efficiently extracted Eu^{3+}, forming the LC. However, the short luminescence lifetime (150–180 μs) and low luminescence yield suggested that such coatings were too permeable to water and nitrate anions to serve as the substitutes for the organic solvents.

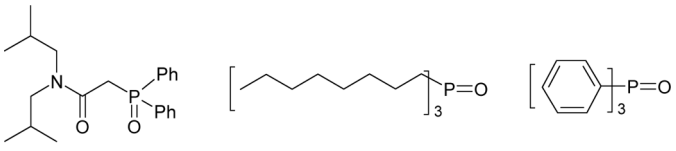
TTA-labeled PAMAM dendrimers were obtained by reacting **12** with **4** in 1:1 DMFA/DMSO, and the conjugate was purified by dialysis. Up to 30% of the 128 amino groups of **12** can be reacted with **4** without the loss of solubility. For polycarboxylic acids, the **5–4** conjugate was amide conjugated using EDC protocol in PBS containing 30 vol% DMSO.

Solution Assay Protocols

EDTA-conjugated cellulose MMs (MagaCell-EDTA, 10 μm, 5 wt%, 50 μmol Ni^{2+} per g of the MMs) from Cortex Biochem and iminodiacetic (IDA) conjugated silica MMs (BcMag-IDA, 1 μm, 2 wt%, binding capacity 55 μmol Ni^{2+} per g of the MMs) from BioClone were used for this assay.



Ca. 10^7 of the MMs suspended in 0.1% Triton X-100 were stirred with 1 mL of Eu^{3+} solution in 10^{-4} HNO_3 for 1 min, and the MMs were magnetically separated. The separated MMs were contacted with 500 μL of 0.1% Triton X-100 solution containing 0.5 mM TTA and 0.5 mM octyl(phenyl)-*N,N′*-di(*iso*-butyl) carbamoyl methyl phosphine oxide (CMPO, 13), (TOPO, 14), or triphenylphosphine oxide (TPO, 15).



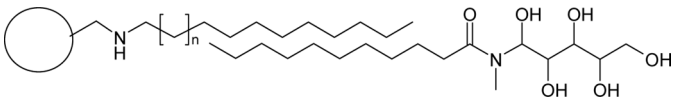
13 **14** **15**

After 1 min stirring, the MMs were magnetically separated, and the LC in the micellar solution was detected using TRLF. See Section 3.1 for further discussion of these assays.

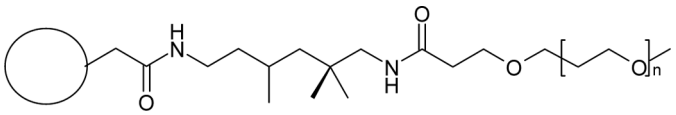
"Supermicelle" Assay Protocols

An adaptation of the solution assays for immobilization of the ions by the MMs is a "supermicelle:" providing a

hydrophobic layer around the MMs into which the LC can be extracted. This layer shields the complex from water impeding the formation of aqua-complexes, in which the luminescence is quenched. The challenge is preventing the aggregation of these hydrophobically coated MMs. Two approaches have been used to address this problem. In the first approach, amino-MMs were reacted with C_{10}-C_{22} alkyl bromides or iodides in methanol/*iso*-propanol mixture saturated with NaOH, by refluxing this reaction mixture for 24 hr. The modified MMs were multiply washed by ethanol with intermittent 1 min sonication, and then washed and suspended in 0.1% solution of MEGA10.



These MMs remained fully suspendable and stable for several months. The most efficient extraction of the LC was observed for C_{18}-alkylated MMs. The second approach was the synthesis of a Triton X-100 like detergent covalently attached to the MM surface. To this end, carboxy-MMs was conjugated with 6 or 8 and the free amino group was reacted with NHS-activated carboxylated PEG_{12} oligomer (Model 22685, Pierce Biotechnology). This conjugate required no detergent for suspension.



In the typical assay, an aliquot of Eu^{3+} solution in 10^{-4} M HNO_3 was added to an aqueous solution containing 0.75 mM TTA and 0.25 mM tri-*n*-butyl phosphate (TBP). These ligands were extracted into the hydrophobic layer at the MM surface and formed the LC there.

"Physisorption" Assay: The Microsphere Impregnation Protocol

In this type of assay, the MMs are covered with a monolayer of the ligand without inducing MM aggregation (Fig. 1a). This can be achieved by maintaining high ionic strength in the MM suspension (5–25% NaCl), at which the sodium cations form a relatively stable complex with the polar P=O group. The repulsion between the ionic atmospheres stabilizes the MMs in such solutions.

The procedure is as follows: a few milligram of the phosphine oxide ligand or 2:1 mol/mol mixture of the ligand with TBP is sonicated for 3 hr at 50°C in 300 mL of 20% NaCl containing 0.01% MEGA10 as the emulsifier. The emulsion is left to sit for 15 min and ≈25 mL near the bottom of the vessel is withdrawn and filtered through a paper filter. 1 mL of the filtrate is added to PMS-20 microspheres washed by 20% NaCl and suspended in 10 mL of saturated NaCl solution. This mixture is vortexed for 10 sec and then sonicated for 15 min at 50°C. The MMs are separated and then suspended in 20% NaCl solution containing 0.01% MEGA10. The cycles of magnetic separation, suspension, and sonication are repeated 5–8 times. The solution of impregnated MMs is stored in 10–20% NaCl containing 0.01% MEGA10 at room temperature. The surface density of CMPO molecules for CMPO-impregnated PMS-20 microspheres was ≈2.7 molecules per nm^2, which is equivalent to a monolayer coverage.

Assay Protocols for Ligand Impregnated and Dressed Microspheres

In a typical assay using MMs with ligand impreganted or covalently attached spacer and ligand groups (Figs. 1b–f), 300 μL of Eu^{3+} solution in 10^{-4} M HNO_3 was mixed with 300 μL of 1–3 mM TTA and 30 μL of 2.5 wt% suspension of the modified MMs. The mixture was stirred gently for 30 sec and the luminescence was measured at intervals of 30 sec, with periodic stirring of the solution. The luminescence fully developed in 1–2 min and then gradually subsided to 50–60% of the initial yield. The solution was magnetized and the luminescence of the supernatant was measured to estimate the contribution from the bulk of the solvent (which was <0.1–1%). At the lowest concentration end, 30 μL of 10 mM Na_2EDTA was added in order to quench the luminescence and acquire the background signal. The magnetically separated MMs were resuspended in 600 μL of 0.5–1.5 mM TTA in 0.1% MEGA10 or a 1:1 mixture of 0.1% MEGA10 and 10% NaCl. For some of the MM designs (e.g., for TBP/CMPO CMPO impregnated MMs the cycle can be repeated 5–10 times before the luminescence begins to decrease. For most of the designs, 2–3 of such cycles do not decrease the TRLF signal by more than 50%.

Alternatively, Eu^{3+} was first extracted by 30 μL of 2.5 wt% microspheres from 300 μL of 10^{-4} M nitric acid solution; the MMs were magnetically separated and then resuspended in 600 μL of 0.5 mM TTA in 0.1% MEGA10 (or 10% NaCl, for "physisorption" assays). This pre-extraction method yielded luminescence that was similar or even higher than the method described above. Using the solution assay on the supernatant, the extraction efficiency of the MMs was simultaneously determined.

RESULTS AND DISCUSSION

Solution Assays

The detection limit of 10^{-13} M Eu^{3+} was achieved using the solution assay method. The sensitivity can be improved further by increasing the volume of the sample

and the contact time. The signal was linear with Eu^{3+} concentration in the concentration range of 10^{-13} to 10^{-6} M. Replacing Triton X-100 with other PEGylated nonionic and even cationic (e.g., cetyl triethylamine bromide) detergents yielded similar results. By contrast, such detergents as sodium dodecyl sulfate, Tween 20, and AOT yield low emission due to the high water content in the micelles. Good results were obtained using carbohydrate derivatives, such as octyl-β-D-glucopyraside and dodecyl-β-D-maltoside. The latter has a low cmc of 0.17 mM and exhibits such strong affinity for phosphine oxides that even water-soluble $Et_3P{=}O$ and $Me_3P{=}O$ form the LC in these micelles. For other micelles, the use of hydrophobic ligands **13–15** was required. While all of the phosphine oxides enhanced the luminescence, the phosphates, diphosphate esters, and phosphonic acids did not yield the LC. The most efficient luminescence enhancing agent was CMPO. Other neutral ligands, such as phenanthroline and its 5-amino derivative also yielded luminescence enhancement, but much lower than the phospho-organic ligands, and no luminescence when this ligand was immobilized at the MM surface. Using 4,4,4-trifluoro-1-(2-naphthyl)-1,3-butadione and benzoylacetylacetone instead of TTA also gave the reduced emission yield, despite their frequent use in immunoassays (16–18). The typical luminescence lifetimes were 650–720 μs.

"Supermicelle" Assay

The typical luminescence lifetime for this type of the assays was 250–350 μs. The performance of the two types of the "supermicelle" conjugates discussed in the previous section was similar, despite the structural differences. The main detriment of this scheme is that TBP forms micelle-like aggregates (35) that extract the luminescent $Eu(TTA)_3$ complex, resulting in background luminescence. While most of the luminescence is from the surface LCs, the contrast ratios between the MMs and the micelles vary between 1:5 to 1:25. Another detriment is nonlinear concentration dependence, which complicates the measurement. Still, reliable determination of $[Eu^{3+}]$ in the 10 nM to 1 μM range has been demonstrated. The luminescence yield rapidly decreases in the presence of the nitrate: the addition of 25 mM NO_3^- decreases the emission yield by 50%. Other assays were considerably less sensitive to the presence of this anion.

It was expected that the impregnation of the "supermicelle" MMs with **13–15** instead of TBP would provide a closer analog of the DELFIA micelle assay. The suspensions of the modified MMs were impregnated by these ligands using sonication of the suspensions/emulsions. This approach was unsuccessful: either the MMs aggregated or the luminescence enhancement was weak, due to the low concentration of the ligands in the surface layer. Using 10–25% NaCl as a solvent allowed the impregnation of the MMs without the aggregation. Subsequent trials demonstrated that no surface modification was required for the impregnation. This observation provided the basis for the "physisorption" assay described below.

"Physisorption" Assay

In this type of the assay, the magnetic microspheres are modified by the formation of a monolayer of a neutral ligand which is stabilized against the aggregation by the high ionicity of the aqueous solution. The LC formed at the surface is in a highly hydrophobic environment, as the observed luminescence lifetime was 350–430 μs (see Table 2). The "physisorption" assay yields the greatest luminescence enhancement among the surface assays that we assessed. The detriment of these assays is that high salinity must be maintained to prevent the aggregation of the MMs. In dilute solutions, there is ongoing aggregation of the MMs that shortens the acquisition of the luminescence signal to a few minutes after mixing the reagents. Another problem is stripping of the metal ions for the subsequent MS analysis. Once the complex is formed at pH 4–7, the re-extraction of Eu^{3+} into the aqueous phase using 0.01–1 M HNO_3 is slow (5–10 min) and incomplete. Upon the increase in the acidity, these MMs begin to aggregate and the LC is trapped inside the aggregates. Since this tendency cannot be countered by addition of detergents (that back extract the ligand molecules into the aqueous phase), covalent attachment of the ligands is preferable, despite the lower luminescence enhancement.

TABLE 2
Relative luminescence yield in a series of trials[a]

MMs	Spacer	Ligand	Lum. signal, arb.u.	Lum. lifetime, μs
AMS-40	–	3a	1320	200
AMS-40	–	1	1850	220
CMS-30	–	3b	2700	280
AMS-30	–	2	1050	220
CMS-30	7	3a	730	213
CMS-30	7	1	2380	275
CMS-30	7	2	790	250
CMS-30	8	2	780	240
AMS-40	9[b]	3b	2150	240
AMS-40	11	2	2800	260
CMS-30	12	1	1270	280
CMS-30	12	2	1070	270
PMS20/TOPO	–	–	2960	310
PMS20/CMPO	–	–	2100	270

[a]The standard assay for 1.5×10^{-8} M (23 ppb) Eu^{3+} and 1.5 mM TTA in 600 μL of 5×10^{-5} HNO_3 with 0.12 wt% of the modified MMs.

[b]$n = 6$.

In addition to CMPO and TOPO, we examined the MMs impregnated by rim decorated calix[4]crown-bound diglycolamide- and CMPO-like groups (36–40) and related tripodal structures (29–31,41). Contrary to our expectations, these extracting agents showed no significant advantage over the CMPO and TOPO, both at low and medium pH.

Covalently Attached Ligands

All of the surface assays based on the covalently attached ligands exhibited linear scaling of the luminescence signal with Eu^{3+} concentration in the solution over a wide dynamic range (2×10^{-10}–2×10^{-5} M). The typical results of trials for relative luminescence efficiency under identical excitation conditions, shown in Table 2. Our conclusion is that clustering of the neutral ligands using branched spacers (Fig. 1d), polymers (Fig. 1e), and dendrimers (Fig. 1f) did not show significant advantages over the simpler designs of Figs. 1b and 1c, either in terms of the extraction efficiency or luminescence enhancement. The greatest enhancement is provided by ''physisorption'' designs (Fig. 1a), although the best covalent-attachment designs Figs. 1b–f are 50–75% as efficient (Table 2). The greatest efficiency among the latter is shown by the MMs in which the ligands were directly attached to the carboxyl and amino groups of the MMs (Fig. 1b); the addition of spacers (Figs. 1c, d) had either a weak effect or a reduced the luminescence yield. The designs in which the polymers/dendrimers were first conjugated to the MMs and then exhaustively labeled by the ligands yielded the largest luminescence enhancement with the longest lifetimes among the conjugates, suggesting efficient shielding of the LC from the solvent. All three ligands (**1–3**) performed equally well, although the conjugates of **2** and **3b** demonstrated greater long-term stability than the conjugates of **1**. Freshly prepared conjugates of **1** yielded 20–50% greater luminescence than the equivalent conjugates of **2** and **3b**.

For PEG modified MM surfaces, the polymer chains were coiled, with the ligands buried inside the PEG globules, as suggested by the fact that the luminescence is enhanced by TTA but not by TTA attached to polymers and dendrimers (which cannot reach the ligand inside the globule). The neutral ligands attached to the dendrimers and polycarboxylic spacers (Figs. 1d, f) were readily accessible.

In all cases, the lifetime of the luminescence did not exceed 350–400 μs, often being as short as 200–250 μs, suggesting the presence of a luminescence quenching ligand. To estimate how many water ligands are involved, we replaced H_2O water by D_2O in extraction of Eu^{3+} by AMS-40-5b microspheres. The isotope replacement resulted in the increase of the lifetime from 216 μs to 295 μs, from which it was estimated, using the method of Nwe et al. (42) that on average 1.5 water molecules are attached to the Eu^{III} ion. Thus, the short luminescence lifetimes are the consequence of relative permeability of the ''hydrophobic'' surface layer (the ligands covered by the detergent) to water. Increasing the hydrophobicity of this layer leads to aggregation of the MMs.

Adapting the Assay to High-Acidity Samples

As the samples of interest for nuclear forensics and nuclear fuel reprocessing frequently involve 0.1–1 M HNO_3 solutions, the assay was adapted for such acidic solutions. For all of the modified MMs, the extraction efficiency dramatically decreases at $pH < 3$, despite the fact that the same neutral ligands are known to extract Eu^{3+} from such solutions on resins (1,2). No surface modification, however, yielded the system capable of such extractions with good efficiency, despite the fact that the others have demonstrated such extractions in 0.1–1 M HNO_3 using impregnated MMs (7–11). These impregnated microspheres, however, were strongly aggregated, suggesting that the extraction occurred into the ''pockets'' of the extractant trapped between the aggregated microspheres. Such aggregates are not suitable for microfluidic manipulation. For fully suspended surface modified MMs, other researchers also reported poor extraction efficiency at low pH, which indicates a systemic problem (12–15).

The likely cause of this inefficiency is that the extraction equilibria in liquid–liquid extractions is determined by two coupled equilibria involving the complexes in the organic and aqueous solvents and at their interface, with the greatest contribution to the overall driving force provided by the equilibrium between the complex at the interface and in the bulk of the organic layer. Without the migration of the complex into the bulk of the organic layer, the overall equilibrium in the acidic solution (in which the complexation to TTA is negligible and the metal ion is complexed by nitrate anions) is shifted towards the aqueous phase.

Two assay strategies were used to counteract this deficiency. In one approach, the solutions were neutralized using the equimolar concentration of the organic base (TRIS) prior to the addition of the MMs. The residual NO_3^- anions quench the luminescence, but the sensitivity decreases only by a factor of 3–4 in comparison to the 10^{-4} M HNO_3 solutions. This approach, however, is problematic from the practical standpoint as other ions present in the sample can hydrolyze during the neutralization, unless stabilized in a complex. In the second approach, the extractions were carried at low pH, the MMs were magnetically separated, washed with 0.1 M imidazole buffer ($pH = 6$), and then suspended in a solution of 1 mM TTA in 0.1% MEGA10. Due to the low efficiency of the extraction in the acidic solutions, the sensitivity of this assay decreased by a factor of 15–20. However, it was still possible to determine 10^{-8} M of Eu^{3+} with the signal-to-noise ratio of five. The MMs that performed best for this type

of assay were PMS-20 microspheres impregnated by 2:1 TBP/TOPO.

Covalently Attached Antenna Groups

As a complementary approach, TTA was covalently attached to the MMs and neutral ligands were added in a micellar solution or attached to a polymer/dendrimer. The luminescence enhancement gradually decreased upon the storage of the MMs due to the slow hydrolysis of the N-S bond. Since the LC in solution involves three TTA anions, surface binding of TTA is not as efficient as neutral ligand binding since the synergistic extraction by several TTA groups is suppressed by the combination of relatively low surface density and partial ionization of the protonated form of the TTA. Consequently the luminescence is limited by the binding capacity of the MMs, which is about 5 μL Eu^{3+} ions in 0.25 wt% solution of AMS-40 microspheres reacted with **4**. The luminescence signal is not linear with the concentration of Eu^{3+}, suggesting complex equilibria involving different types of clustered TTA groups. In the 10^{-8}–10^{-6} M Eu^{3+} range, this assay gave 5–20 times lower luminescence yield than the assays discussed in the previous section. However, at the low end of the concentration range, 10^{-10}–10^{-8} M, the luminescence yields from these two types of the assays were comparable and the emission was linear with [Eu^{3+}]. An example of the TRLF kinetic is given in Fig. 2.

We sought to increase the luminescence yield by clustering the TTA groups, but the LC yield did not increase appreciably when several TTA groups were attached to tripods, polymers (up to 15 TTA molecules per chain), and dendrimers (up to 30 TTA molecules per dendrimer), which is likely to be due to the fact that keto-enol equilibrium is shifted to the keto-form in the pH range where Eu^{3+} does not hydrolyze. The relatively high concentration of the protonated form of TTA in the solution provides sufficient concentration of the TTA base for binding to Eu^{3+}, whereas the covalent binding of the TTA groups interferes with the assembly of the $Eu(TTA)_3$, which requires the presence of three TTA bases at a single site. In the absence of the charge-compensating TTA groups, the NO_3^- anions in the solution and $-SO_3^-$ anions at the surface remain bound to the Eu^{3+} ions resulting in the formation of complexes with low luminescence yields and short luminescence lifetime that evade TRLF detection.

Ion-Exchange Assay

The previously examined assays are suitable for the detection of luminescent ions only. The ion-exchange assay examined in this section does not rely on the antenna effect; rather it uses the ion exchange properties of the sulfonated MMs. The MMs are preloaded with a luminescent ion, such as Eu^{3+}. When this ion is exchanged with a nonluminescent ion in a solution, the luminescent ion is released into the bulk, where it is detected using one of the assays described above. Let $[L]$ be the concentration of the free ion-binding ligand at the MM surface, $[M_{1,2}]$ and $[m_{1,2}]$ are the free ion and total concentrations of the luminescent (1) and non-luminescent (2) ions and $[LM_{1,2}]$ the concentration of the ligand-bound ion.

Then the concentrations can be determined from

$$[M_{1,2}] = m_{1,2}/\big(K_{1,2}[L] + 1\big), \tag{1}$$

$$[L] + [M_1] + [M_2] = [L]_0 + [m_1] + [m_2], \tag{2}$$

where $K_{1,2}$ are the corresponding binding constants. Solving these equations first for Eu^{3+}, using the experimental data on extraction of Eu^{3+}, allows one to determine the maximum capacity $[L]_0$ for these ions and the binding constant K_1 and use these parameters to estimate the optimum loading $[m_1]/[L]_0$ to obtain the widest linear range of the plot of $[M_1]$ vs. $[m_2]$. For $K_1 \approx K_2$, numerical simulations indicate this optimum is at $[m_1]/[L]_0 \approx 0.65$.

The MMs differ in their capacity as ion exchangers, and this capacity can be tuned by changing the ionic strength. For the standard 0.25 wt% solution of the MMs in 10^{-4} M HNO_3 containing 150 nM Eu^{3+}, the extraction efficiency of unmodified PMS-20, AMS-40, and CMS-30 microspheres is 90%, 70%, and 98%. In the identical solution containing 10% NaCl, this extraction efficiency is reduced to 43%, $\approx$0%, and 17%, respectively. The maximum loading capacity $[L]_0$ of PMS-20 microspheres for Eu^{3+} in these solutions was estimated at 0.24 mM. Other types of the MMs also demonstrated high capacity at low ionicity, but only functionalized microspheres, such as MagaCell-EDTA and ligand-conjugated microspheres, exhibited high extraction efficiency at high ionicity. For 0.25 wt% of AMS-40 microspheres in 10^{-4} M HNO_3, $[L]_0 \approx 6\,\mu M$ and $K_1[L]_0 \approx 19$. For good reproducibility, the presence of a nonionic detergent in the solution was required, as the MMs loaded with ions tended to aggregate otherwise. For 0.07% CMS-30 in 10^{-4} M HNO_3 containing 0.1% Triton X-100, $[L]_0 \approx 4.6\,\mu M$ and $K_1[L]_0 \approx 60$. For the assay shown in Fig. 3, this solution was equilibrated with 4.3 μM Eu^{3+} and 250 μL of the suspension mixed with 250 μL of Gd^{3+} in 10^{-4} M HNO_3. After 1 min stirring, the suspension was magnetized and 50 μL of the supernatant mixed with 1.5 mL of 0.5 mM TTA and 0.5 mM CMPO in 0.1% Triton X-100. The luminescence of the LC was determined and compared to the luminescence of the standard Eu^{3+} solution. As seen from the figure, the luminescence signal (converted to [Eu^{3+}]) is linear with [Gd^{3+}] to 30 μM, although there is a weak luminescence background from free Eu^{3+} ions in the solution due to the extraction equilibrium. The latter is easily taken into account by a separate measurement, and so the range of reliable detection is 2–30 μM (~0.5–6 $[L]_0$). Our

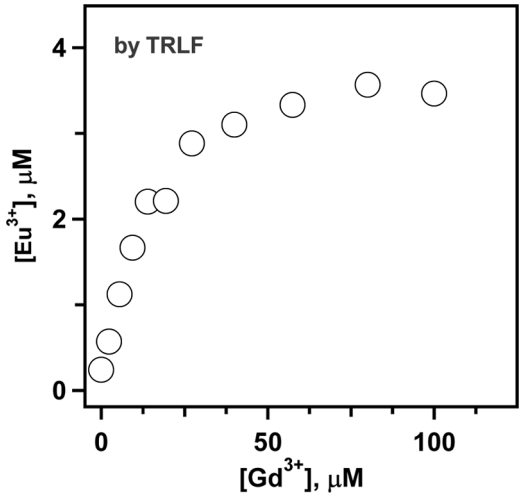


FIG. 3. The Eu^{3+}/Gd^{3+} ion exchange assay for PMS-20 microspheres loaded with Eu^{3+}. The concentration of Eu^{3+} in the aqueous solution was determined using a DELFIA-like solution assay.

simulations indicate that for Gd^{3+}, the binding is weaker than for Eu^{3+}, $K_2[L]_0 \approx 1$. As these equilibria do not depend on the concentration of the MMs, this assay can be adapted to lower concentrations of Gd^{3+} ions by decreasing the concentration of the CMS-30 microspheres and Eu^{3+} ions accordingly.

These measurements can also be carried out using surface and solution assays examined in the previous sections, by diluting the supernatant containing the released Eu^{3+} ions and then performing one of these assays. Therefore, by combining the ion exchange MMs and the mixed-ligand MM assays, it is possible to detect other ions than Eu^{3+}. Although the identity of these ions cannot be ascertained from the assay, their concentration can be estimated.

Mass Spectrometry

Three types of the MS have been used in our study: electrospray ionization (ESI), inductively coupled plasma (ICP), and laser desorption ionization (LDI) with 337 nm photoexcitation. Hereafter, the concentration of Eu^{3+} is given in ppb (1.5 ppb $= 10^{-9}$ M).

These techniques were first evaluated for a DELFIA-like based assay. Since ESI MS is a soft ionization technique, the LC lost one of the TTA ligands, but was not broken apart, so the isotopes from all the elements present in the complex needed to be accounted to calculate the mass present from the $\{Eu(TTA)_2(CMPO)_2\}^+$ ion. Figure 4 displays the expected isotopic distribution plot compared to that obtained experimentally. It is clear that there is good agreement between expected and observed results. We next evaluated the ICP MS and LDI MS techniques. Both of these techniques were able to fragment the LC complex and detect isolated Eu^{3+} ions. The isotope ratios for the latter

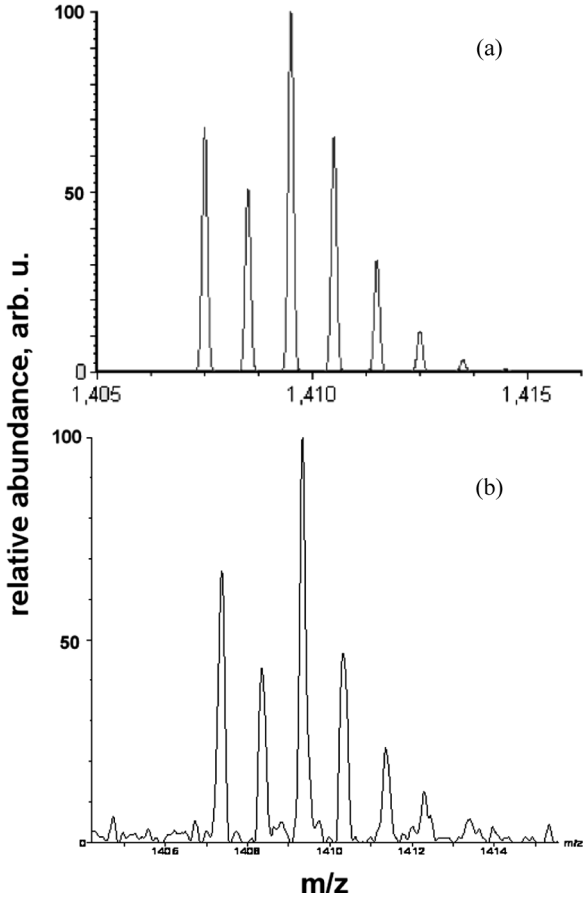


FIG. 4. Electrospray ionization MS results for a solution assay (Section 3.1). (a) Calculated isotope distributions. (b) Mass spectrum of $\{Eu(TTA)_2(CMPO)_2\}^+$.

were determined with 0.1% accuracy at 5.6 ppb concentration with ICP and at 114 ppb with LDI.

We next examined the MM assays using AMS-40-**3a** microspheres. The extracted Eu^{3+} was stripped from the MM surface using 1 M HNO_3, the MMs were magnetically separated, and the supernatant solution was analyzed using ICP MS. For 8 ppb Eu^{3+}, the recovery of Eu^{3+} was 70%. When LDI MS was used, there was no need for acid stripping prior to the MS analysis. The assay was formed at 2 ppb and 50 ppb Eu^{3+} in solution and the magnetically separated (wet) microspheres were analyzed directly. Bare Eu^{3+} ions were observed at both of these concentrations. Using the signal to noise ratio of the 2 ppb Eu^{3+} measurement, the lowest detection limit was estimated at 0.1 ppb. The high sensitivity of this LDI MS method as compared to acid stripping ICP MS measurements results from concentrating of the Eu^{3+} by the MMs surface.

CONCLUSION

We have demonstrated several approaches for magnetic extraction, sensitive fluorometric detection, and subsequent

mass spectroscopy analysis of the lanthanide ions using mixed ligand complex formed at the surface of chemically modified magnetic microspheres (MMs). Luminescent lanthanide ions, such as Eu^{3+} (<1 ppb), can be detected directly using the antenna effect. Nonluminescent ions can be detected using the same scheme by taking advantage of ion-exchange properties of the sulfonated polystyrene microspheres.

These results pave the way for microfluidic devices for analytical chemistry and nuclear forensics using the suspendable MMs as the carriers of metal ions.

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CHEMICAL AND INSTRUMENT SOURCES

B&W Tek (Newark, Delaware)
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Stanford Research Systems (Sunnyvale, California)
Pierce Biotechnology (Rockford, Illinois)
Quantel USA (New York, New York)
Tektronix (Beaverton, Oregon)

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