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Preparation of Metal-*p*-aminobenzyl-DOTA Complex Using Magnetic Particles for Bio-tagging in Laser Ablation ICP-MS

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Metal-*p*-NH_2-*Bn*-DOTA (paraammionobenzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid: ABDOTA) complex was synthesized and purified for bio-tagging to quantify biological target materials using laser ablation (LA)-ICP-MS. Since the preparation of a pure and stable tagging complex is the key procedure for quantification, magnetic particles were used to purify the synthesized metal-ABDOTA complex. The magnetic particles immobilized with the complex attracted to a permanent magnet, resulting in fast separation from free un-reacted metal ions in solution. Gd ions formed the metal-complex with a higher yield of 64.3% (± 3.9% relative standard deviation (RSD)) than Y ions, 52.3% (± 2.5% RSD), in the pH range 4-7. The complex bound to the magnetic particles was released by treatment with a strong base, of which the recovery was 81.7%. As a reference, a solid phase extraction (SPE) column packed with Chelex-100 resin was employed for separation under similar conditions and produced comparable results. The tagging technique complemented polydimethylsiloxane (PDMS) microarray chip sampling in LA-ICP-MS, allowing determination of small sample volumes at high throughputs. For application, immunoglobulin G (IgG) was immobilized on the pillars of PDMS microarray chips and then tagged with the prepared Gd complex. IgG could then be determined through measurement of Gd by LA-ICP-MS. A detection limit of 1.61 ng/mL (± 0.75% RSD) for Gd was obtained.

Key Words : Magnetic particle, Purification of DOTA complex, Bio tagging, Microarray chip, LA-ICP-MS

Introduction

Laser ablation (LA)-ICP-MS can be very effective and advantageous for biological analysis, allowing high throughput analysis with limited sample volumes.[1-4] However, some limitations remain that hamper its wider application in addition to intrinsic problems, such as fractionation and the lack of standard materials. LA-ICP-MS with microarray chips has aroused recent attention because it allows high throughput and ultra trace sampling. Such sampling involves the immobilization of a sample on the chip followed by metal tagging.[5-9] For this, the tagged material should be firmly attached and stable against washing and storage without sample loss. This can be achieved through chemical bonding rather than intermolecular interactions.[6,7]

Synthetic macrocyclic and acyclic ligands with amino and carboxylate groups as metal-binding moieties have been employed in biomedical and radiopharmaceutical applications such as magnetic resonance (MR), positron emission tomography (PET) imaging and the antibody-targeted radiation therapy (radioimmunotherapy, RIT) of cancer.[10] Macrocyclic 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) is one of the most widely used macrocyclic ligands for the development of new metal-based imaging agents as it can provide the desire stability.[6,11,12] It forms complexes with Ln (III) ions that are thermodynamically stable and kinetically inert[12,13]; it is used for the diagnosis and therapy of tumors in nuclear medicine[14] and in MRI contrast agent Dotarem.[15-17] Both its formation and dissociation rates are much lower than those of acyclic polyamino polycarboxylates, such as EDTA.[18,19] Therefore, the un-reacted free metal should be effectively eliminated after reaction in order to reduce quantification error.[20]

For the purification of the metal complex from the free metal ions, solid phase extraction (SPE) is typically used and offers a number of important benefits.[21] It reduces solvent use and exposure, and also lowers the disposal costs and extraction times of sample preparation.[22,23] Particularly, iminodiacetic acid resins, *e.g.*, polystyrene-structured Chelex-100, which can form complexes with metal ions, have been used in the extraction and pre-concentration of trace metals from aqueous complex matrices.[24-26]

An alternate approach suggested in this work uses magnetic particles that are being used increasingly across several biomedical fields. Upon functionalization for specific binding, magnetic particles and their targets can be conveniently positioned *in vitro* and *in vivo* by magnetic fields.[17] They also have been used in inorganic separations. Attachment of EDTA-like chelators to magnetic particles produced a magnetic reagent capable of the rapid removal of heavy metals from solutions of contaminated water.[27]

In this work, *p*-NH_2-*Bn*-DOTA (ABDOTA) was prepared as a tagging material because it contains amine groups that can react with the carboxylic groups of magnetic particles and biomaterials, forming covalent amide bonds.[2,27] After immobilization on the magnetic particles, ABDOTA formed

a complex with lanthanide ions and could be separated from un-reacted free metal ions using a permanent magnet.[28] Gd-ABDOTA bound to magnetic particles was recovered by treatment with a strong alkali. In applicability test of the prepared complex, immunoglobulin G (IgG) antibody was immobilized on the pillars of PDMS microarray chips for sampling and then, tagged with the Gd-ABDOTA. The concentration of IgG was estimated from the determination of Gd using LA-ICP-MS.

Experimental

Synthesis and Purification of Gd-ABDOTA Complex. Gd-ABDOTA was synthesized by mixing 100 g/mL Gd ions with 32 g/mL *p*-NH_2-*Bn*-DOTA (at a 10:1 in molar ratio) in 20 mL D.I. water. Since the metal-DOTA complexes formed slowly, high temperature and pressure were preferred to expedite the chelation.[20] The mixture was reacted for 30 min in an autoclave at 121 °C. After the reaction, excess metal ions were removed by an exchange column. The column was prepared by packing 1 g iminodiacetic resin (Chelex-100, Sigma-Aldrich Chem.) in its sodium form in a polyethylene column with 20 m frits. Prior to use, the Chelex-100 resin was vortexed with 1 M HCl for 6 h and reacted with 1 M NaOH in a Teflon beaker, and finally washed with de-ionized water. The complex mixture of 4 mL was transferred to the Chelex-100 column for purification. A flow rate of 0.3 mL/min was maintained by keeping the manifold of the column under a vacuum of 25 mmHg. Gd in the complexes was determined by ICP-MS to estimate the extent of reaction. The overall experimental procedure is summarized in Figure 1.

Metal-ABDOTA Complexing with Magnetic Particles. Amine-functionalized DOTA was immobilized on superparamagnetic particles (Dynabead®, MyOne™ Carboxylic Acid, 1.05 μm, Invitrogen) and then chelated with Gd and Y ions, forming metal-ABDOTA complexes. The un-reacted free metal ions in the supernatant were determined by ICP-MS and the magnetic particles holding the complexes were captured by a permanent magnet. After removing the free metal ions by washing, metal-ABDOTA complexes were detached for tagging by cleavage of the amide bond using a strong base, KOH. The experimental procedure is shown in Figure 2.

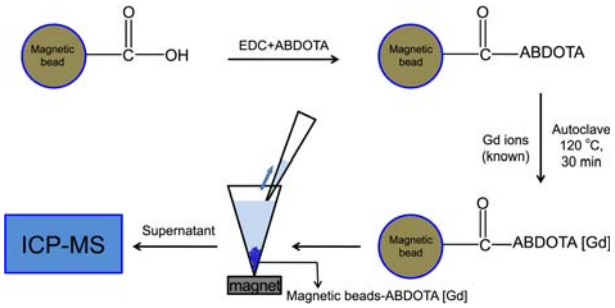

Figure 1. The purification of Gd-DOTA complex using a Chelex column.

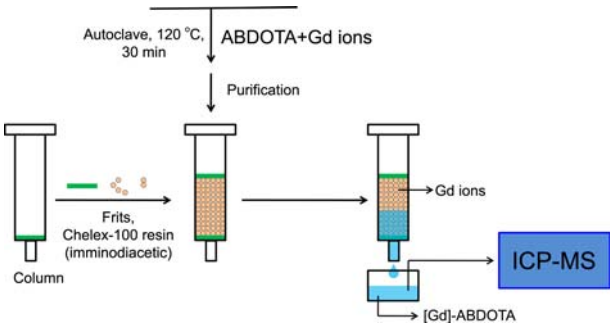

Figure 2. The immobilization of Gd-ABDOTA complexes on magnetic particles.

Immobilization of ABDOTA on Magnetic Particle and Chelation. To immobilize ABDOTA, 600 μL of 25 mM MES buffer (pH 6), prepared from MES (2-morpholinoethansulfonic acid monohydrate, 99%, Merck), was added to 600 μL (*ca.* 6 mg) magnetic particles functionalized by carboxylic groups and washed twice after vortexing for 10 min. To activate the carboxylic groups, EDC (*N*-(3-dimethyl amino propyl)-*N*-ethyl carbodiimide hydrochloride, 99%, Sigma-Aldrich Chem.) and NHS (*N*-hydroxy succinimide, 98%, Sigma-Aldrich Chem.) solutions were prepared by dissolving 50 mg of each in 1 mL of the cold MES buffer. The washed magnetic particles were then reacted with 100 μL EDC for 10 min and vortexed in 100 μL of NHS for 1 h. After being mixed with 2 mg ABDOTA in 120 μL MES buffer, the activated magnetic particles were vortexed for 2 h. The product was washed three times with 600 μL of de-ionized water and stored at 2-8 °C. To detach ABDOTA from the magnetic particles, strong base, 11.7 M KOH, was added to the solution and reacted for 2 h. The mixture was then adjusted to pH 8.3 with 1% (v/v) HNO_3. For further procedure, potassium ions were removed from the SPE column, if necessary.

Immobilization of IgG on PDMS Microarray Chips and Tagging with Metal-DOTA. Array-type microchips, *ca.* 4 × 4 mm in size, were made from polydimethylsiloxane (PDMS) by a typical manufacturing process.[29] After preparing a polymer mask film with 4 × 4 arrays of 150 μm diameter holes, using a 213 nm Nd:YAG laser (LSX-213, Cetac, Omaha, USA), PDMS monomer (SYLGARD 184, Dow Corning) with curing agent was poured onto the mask film and polymerized on the base plate (100 mm × 100 mm) by degassing in a vacuum oven and curing at 70 °C for 2 h. The mask film was then removed after being treated with oxygen plasma to render the exposed PDMS pillars hydrophilic.[30,31]

Since direct binding of IgG on the PDMS chip can disrupt its reactivity and reduce the immobilization efficiency, a linker was inserted between the pillar and IgG prior to immobilization. 1 mL GPTMS (3-glycidoxypropyl-trimethoxysilan, 98%, Sigma-Aldrich) was deposited on the chip by chemical vapor deposition (CVD) in a vacuum of 76 torr vacuum at 100 °C for 2 h. Then, the chip was washed with ethyl alcohol (99.9%, Duksan, Korea) and dried with flowing Ar gas. A 300 μg/mL portion of IgG (from human serum,

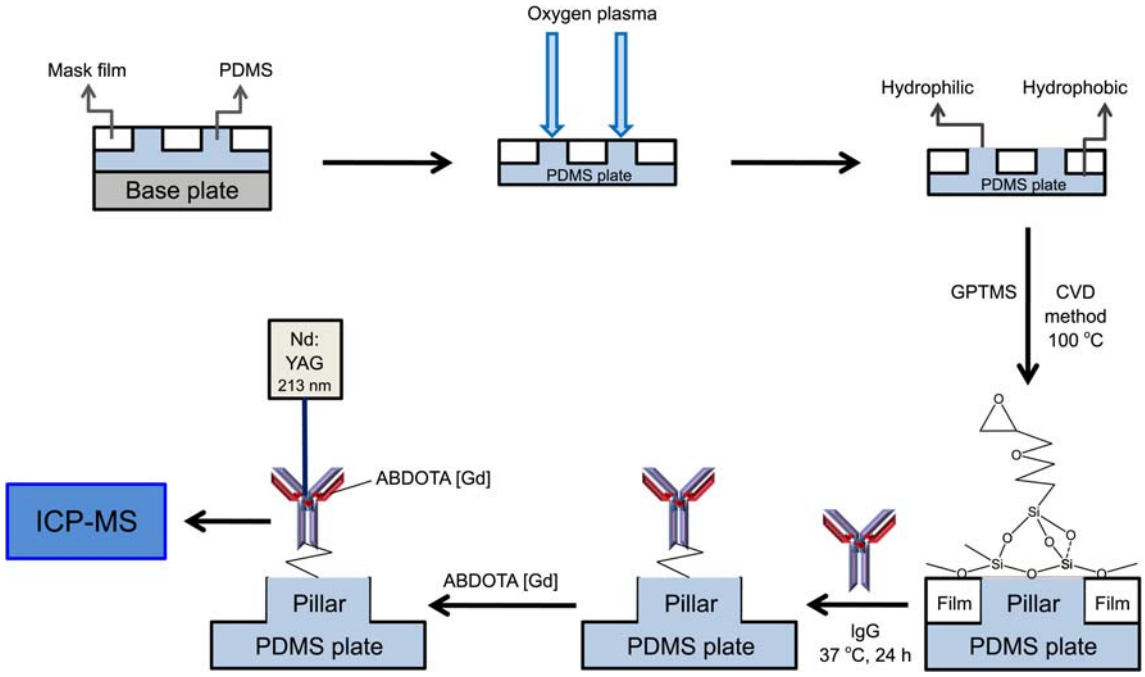

Figure 3. The determination of IgG immobilized on PDMS microarray chips by LA-ICPMS.

Table 1. The operating conditions of LA-ICP-MS

Instruments	Operating Conditions
ICP-MS	Perkin-Elmer, DRC-e
Nebulizer gas flow	1.2 L/min
Auxiliary gas flow	1.0 L/min
Plasma gas flow	17.0 L/min
Lens voltage	7.0 V
RF power	1350 W
Scan mode	Peak Hopping
Laser ablation	Cetac, LSX-213, Nd:YAG laser. 20 Hz
Wavelength	213 nm
Beam diameter	150 μm
Energy	4 mJ/pulse, Single shot

95%, Sigma-Aldrich) in PBS buffer (× 10, Biotech) was immobilized on the pillars of the microarray chips during incubation for 24 h. The completed chips were washed three times with PBS buffer.

The tagging of the prepared Gd-ABDOTA complex to IgG on the chip was similar to the immobilization of *p*-NH_2-*Bn*-DOTA on the magnetic particles. Purified Gd-ABDOTA of 10, 50, 100, 500 ng/mL in PBS buffer was sprayed on to the PDMS microarray chips after activating the COOH groups using EDC and NHS solutions, and then incubated for 24 h at 37 °C. After washing the chips three times with PBS buffer, the target metal on the pillars was determined by LA-ICP-MS. LA-ICP-MS is outlined in Figure 3 and Table 1, respectively.

Results and Discussion

Gd-ABDOTA Complex. As ABDOTA was used as the chelating ligand in biological tagging, the formation of metal-ABDOTA complexes is very important as their determination was directly related to the concentration of the target protein, *i.e.*, IgG in this work. For DOTA, production yield was reportedly close to 100% and the complex was sufficiently stable at the high temperature required to initiate the reaction.[10,15] However, the procedure to obtain the production yield was not performed quantitatively and unclearly. Since Gd ions were added in excess for the synthesis in this experiment, the complex required purification prior to use because the presence of un-reacted metal ions can enhance background signals significantly and interfere with quantitative measurement. Although both targets have similar positive charges, their different mobilities and sizes can make separation possible.[22,24,25] A Chelex-100 column was used for the separation by capturing metal ions and eluting the complex.

When 10 ng/mL Gd was reacted with ABDOTA, 5.89 ng/mL (± 3.08% RSD) was found in the complex by ICP-MS. As a blank test, Gd^{3+} without ABDOTA was eluted and produced a background equivalent to 0.21 ng/mL (± 35% RSD). This indicates that almost all the un-reacted Gd^{3+} ions were retained by the Chelex-100 column and did not interfere with the measurement of Gd-ABDOTA in the eluted solution. The collected Gd-ABDOTA complex was used in subsequent tests.

Immobilization of *p*-NH_2-Bn-DOTA on Magnetic Particles. An alternate separation technique involves magnetic particles. Advances of surface modification technique have allowed ABDOTA to be immobilized on the functionalized surfaces of the particles. Their magnetic properties can facilitate the selective extraction from the matrix of the particles immobilized with target materials. In this work, ABDOTA was immobilized on magnetic particles and then separated from

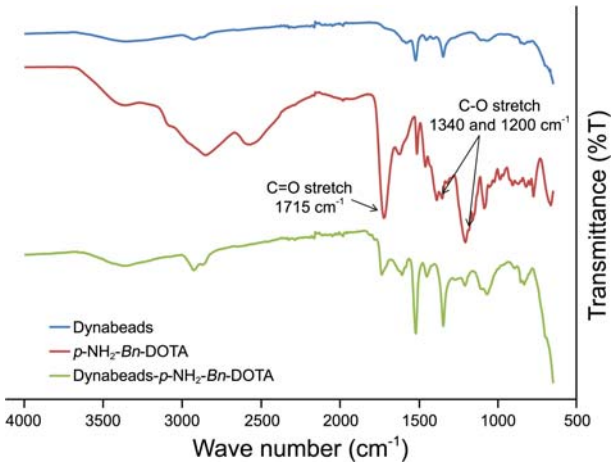

Figure 4. FT-IR spectrum of magnetic particles coated with ABDOTA.

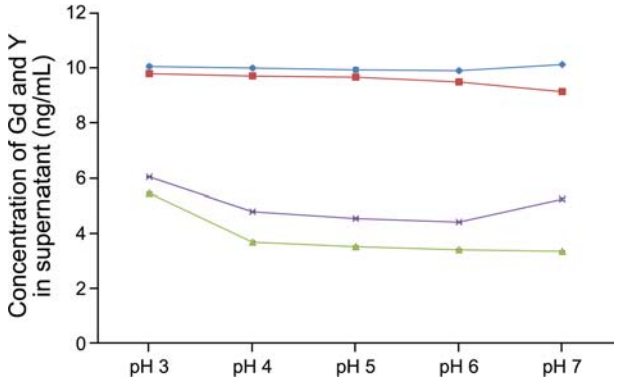

Figure 6. Concentration change of Gd and Y in supernatant with pH change: (a) Y in supernatant reacted with Dynabead (-◆-). (b) Gd in supernatant reacted with Dynabead (-■-). (c) Y in supernatant reacted with Dynabead-*p*-NH_2-*Bn*-DOTA (-×-). (d) Gd in supernatant reacted with Dynabead-*p*-NH_2-*Bn*-DOTA (-▲-).

un-reacted free Gd^{3+} ions in solution using a permanent magnet. The immobilization was achieved by reacting carboxylic acid groups in Dynabead® (polystyrene based magnetic particle) with amine groups in ABDOTA in the presence of EDC. The resulting covalent amide bonds were sufficiently stable for further reaction at 120 ºC. The presence of ABDOTA on the magnetic particles was confirmed by FT-IR (Fig. 4). ABDOTA showed C=O stretching peaks near 1715 cm^{-1} and C-O absorption peak at 1340 and 1200 cm^{-1}. Double bonds of benzene groups and C-N peaks were identified by absorptions at 1580 and 1340 cm^{-1}, respectively. Other peaks from alkyl groups were interposed with peaks originating from the polystyrene substrate of Dynabead®.

Figure 5 shows SEM images of the particles before and after immobilization. The average diameter of 50 particles increased from 1.061 μm to 1.082 μm indicating the presence of ABDOTA on the particles.

Purification of Metal-ABDOTA Complexes Using Magnetic Particles. ABDOTA functionalized magnetic particles were reacted with 10 ng/mL (1:10 molar ratio) Gd and Y ions in batches and excess free metal ions were separated using a permanent magnet. Magnetic particles collected at the surface of the vial, while un-reacted metal ions remained in the supernatant. The un-reacted metal ions were quantitatively determined by ICP-MS. Since the stability of the metal-DOTA complexes depended on pH, the metal ions were determined at various pH values from 3 to 7 (Fig. 6).

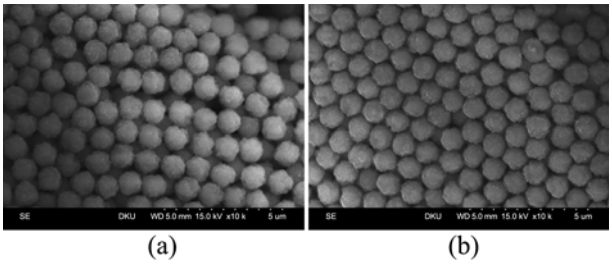

Figure 5. SEM images of magnetic particles: (a) before immobilization and (b) after coating with ABDOTA.

The blank tests of Gd and Y, where metal ions were reacted with Dynabead® at room temperature, showed no concentration reduction of the 10 ng/mL metal ion. However, after the metals reacted with Dynabead® immobilized with ABDOTA, their concentration reduced significantly due to the formation of metal complexes. The concentration of Gd and Y in the supernatants did not change greatly at pH values between 4 and 6. However, at pH 3 both metal ions' concentrations increased as they were released from the complexes. The Y complexes were less stable than the Gd complexes at pH 7. The average formation efficiencies in the pH ranges 4 to 6 were 64.3% (± 3.9% RSD) and 52.3% (± 2.5% RSD) for Gd and Y, respectively. From these data, the equivalence of ABDOTA on the magnetic particles was estimated to be 6.3×10^{-11} mole per 0.2 mg particle. In subsequent tests, metal-ABDOTA was prepared for tagging at pH 5.

The immobilized Gd-ABDOTA complexes could be detached from the magnetic particles by cleaving the amide bond using a strong base, KOH, as the metal complexes are stable in such basic conditions. This treatment resulted in 88.5% detachment of the complex from the particles and was used in the tagging tests.

Application to Determine IgG Immobilized on PDMS Microchips by LA-ICP-MS. As discussed above, LA-ICP-MS with microarray chip sampling is useful in biological analysis, where small sample volume is a limiting factor. The tagging technique is suitable for use with microarray chip sampling in LA-ICP-MS. In this work, IgG was immobilized on a PDMS microarray chips and then tagged by the prepared Gd-ABDOTA. Figure 3 outlines the whole analytical process including sampling and tagging. The linker helped to immobilize the IgG and improve its functionality for tagging.

The various functional groups immobilized on the chip were identified by Fourier transformation -infrared absorption spectrometry (FT-IR), (Fig. 7). The spectrum of epoxy showed characteristic small peaks at 2897 cm^{-1} and 2834 cm^{-1}, corresponding to the sp^3 C-H stretching of alkyl groups.

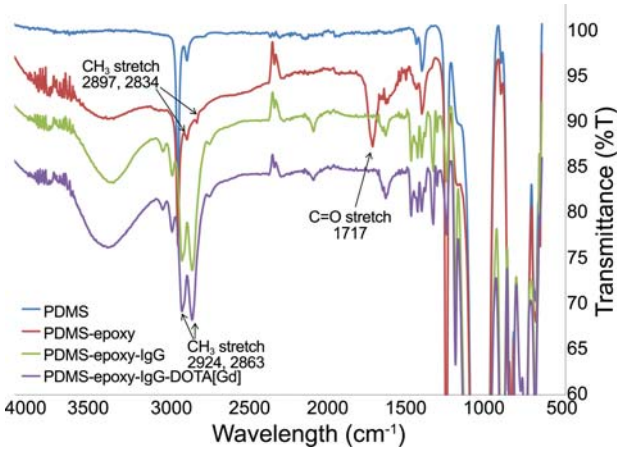

Figure 7. FT-IR spectra of IgG and Gd-DOTA on PDMS microchips: (a) PDMS only, (b) PDMS coated with epoxy linker, (c) PDMS-epoxy coated with IgG, and (d) PDMS-epoxy-IgG tagged with Gd-DOTA.

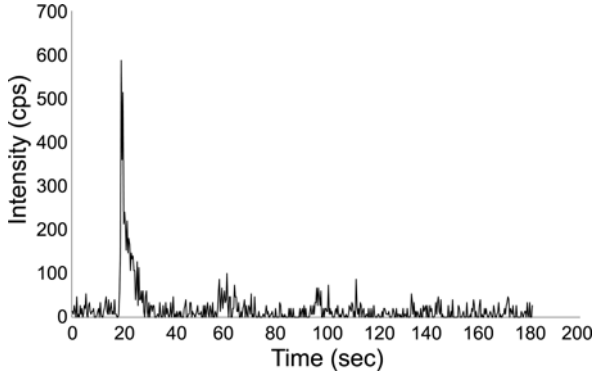

Figure 8. LA-ICP-MS spectrum of Gd on microarray chips for IgG determination.

The medium peak at *ca*. 1717 cm^{-1} is characteristic of epoxy. Therefore, these peaks indicate the presence of epoxy linker on the PDMS chip. When IgG was immobilized on epoxy coated PDMS, these peaks disappeared and characteristic peaks of alkyl groups appeared at 2924 cm^{-1} and 2863 cm^{-1}. Since IgG is a relatively large molecule compared with ABDOTA, no great differences were observed in the FT-IR spectra when the Gd-ABDOTA was tagged.

The presence of IgG was detected through the equivalent measurement of Gd by LA-ICP-MS (Fig. 8). For the measurement, single shot mode laser ablation was employed because the layer was sufficiently thin. A clear Gd peak was observed upon the first pulse, but subsequent peaks were significantly reduced and barely detectable after the second. The concentration of Gd was determined by integrating the areas of all the peaks. Based on standard calibration, a linear regression coefficient of 0.9923 (supplementary figure) and limit of detection of 1.61 ng/mL (± 0.75% RSD) for Gd were obtained from three repeated measurements.

Conclusion

Magnetic particles were used to prepare Gd-ABDOTA and the prepared complex was used to tag a biological sample for its determination using ICP-MS. The amine functionality in ABDOTA aided for the immobilization of the particles. This technique provides a novel, convenient and fast approach for purifying and recovering tagging materials from unwanted interferences. In addition, immobilization of IgG on PDMS microarray chips was tested for sampling and was compatible with LA-ICP-MS. This sampling technique can be extended to analyze biological samples of limited volume although separate immobilization procedures should be developed. Although laser ablation for sample introduction produced relatively poor reproducibility, compared to nebulization, it can be improved by the microarray chip sampling developed in this work through ensemble averaging effect based on repeated measurements.

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