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Versatile immunoassays based on isomagnetophoresis

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Experimental Section

Materials

Goat anti-rabbit IgG and normal rabbit IgG-biotin were purchased from Sigma–Aldrich (St. Louis, MO) and Santa Cruz Biotechnology, Inc (Santa Cruz, CA), respectively. Proteins for the isomagnetophoretic immunoassays using three types of breast cancer biomarkers such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) were all purchased from R&D Systems (Minneapolis, MN). MCF-7 and SK-BR-3 breast cancer cell lines were provided from ATCC (Manassas, VA) and Korean cell line bank (Seoul, Korea), respectively. Superparamagnetic nanoparticle solution was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Streptavidin/anti-biotin were conjugated with magnetic nanoparticles (MNPs) which consist of iron oxide with a diameter of about 50 nm, including polymer coating and proteins on their surface. 10 μm carboxylated microbead for isomagnetophoretic immunoassay of rabbit IgG-biotin and 6 μm fluorescent microbeads (yellow-green: excitation-441 nm, emission-486 nm; yellow-orange: excitation-529 nm, emission-546 nm; bright blue: excitation-360 nm, emission-407 nm) for that of breast cancer biomarkers were bought from Polysciences, Inc (Warrington, PA). Gadolinium paramagnetic diethylenetriamine pentaacetic acid (Gd-DTPA) solution and Tween 20 were obtained from Sigma–Aldrich.

Design and fabrication of an isomagnetophoretic immunoassay microchannel

A chemical gradient in solution plays an important role in a wide range of biological and chemical applications.[1–7] During isomagnetophoresis, the gradient of Gd-DTPA solution is a key factor and should be maintained through an entire microchannel for a long time. Considered for this aspect, the microchannel for isomagnetophoresis was designed almost symmetrically for generating a linear concentration gradient.[6] The microchannels were fabricated by using a conventional poly(dimethylsiloxane) (PDMS) (Sylgard 184; Dow Corning, Midland, MI) molding process, and Ni microstructures were electroplated on a Pyrex glass wafer. Detailed process was followed according to the previously reported method.[8] Briefly, the negative photoresist SU-8 2025 (MicroChem Corp., Newton, MA) was used for a PDMS mold. The isomagnetophoretic immunoassay microchannel was designed to have one main microchannel

for high concentrations of Gd-DTPA solution with high magnetic susceptibility, the other microchannel for low concentrations of Gd-DTPA solution with low magnetic susceptibility, and nine microbridges which connect two main microchannels for generating a stable Gd-DTPA solution gradient without considering flow rate change. The inlet 1 for microbead and inlet 2 for high concentration of Gd-DTPA are connected to the upper microchannel and inlet 3 for the low concentration of Gd-DTPA is connected to the lower microchannel. The selected high concentration of Gd-DTPA solution containing the reacted microbeads is injected into inlet 1, and the same concentration of Gd-DTPA solution is injected into inlet 2. Meanwhile, low concentration of Gd-DTPA solution is injected into inlet 3 which is connected to the lower microchannel. Low concentration of Gd-DTPA solution flows from lower microchannel to upper microchannel through nine microbridges so that a Gd-DTPA solution gradient is formed. The height and width of the main microchannel were 24 and 300 μm, respectively. The width and length of the microbridges were 20 and 100 μm, respectively. The outlet detection region at the rear of upper microchannel is positioned for measuring the position of microbeads and its width is 1 mm.

Selection of the concentration of Gd-DTPA solution for the highest sensitivity

As mentioned in the communication text, we decided the concentration range of Gd-DTPA solution for detecting the infinitesimal quantity of protein prior to the isomagnetophoretic immunoassay. In the isomagnetophoretic immunoassay scheme, a small quantity of MNPs bind to the microbeads for analysis of the infinitesimal protein. Therefore, we calculated the concentration of Gd-DTPA solution whose magnetic susceptibility has the magnetic susceptibility value of a polystyrene microbead using eq S1.

$$\chi_{\mathrm{sol}} = \frac{V_{\mathrm{Gd-DTPA}}}{V_{\mathrm{total}}}\chi_{\mathrm{Gd-DTPA}} + \frac{V_{\mathrm{water}}}{V_{\mathrm{total}}}\chi_{\mathrm{water}} = (0.35C - 9.04)\times 10^{-6}\ (\mathrm{SI}) \qquad \text{(S1)}$$

where $\chi_{\mathrm{Gd\text{-}DTPA}}$ and χ_{water} are magnetic susceptibility of Gd-DTPA solution and DI water, respectively, C (mM) is the concentration of Gd-DTPA solution, V is the volume of respective solution, and χ_{sol} is the magnetic susceptibility of solution analyzed. Because the magnetic susceptibility of a polystyrene microbead is -8.21×10^{-6} (volumetric χ, SI), the concentration of Gd-DTPA solution with the same magnetic susceptibility is calculated as 2.37 mM. Therefore, the magnetic susceptibility gradient was generated by DI water and 10 mM of Gd-DTPA solution for analysis of infinitesimal protein.

Magnetic susceptibility of a Gd-DTPA solution gradient in an isomagnetophoretic immunoassay microchannel.

To analyze the magnetic susceptibility of a Gd-DTPA solution gradient in an isomagnetophoretic immunoassay microchannel, we fabricated the microchannel whose width is 300 μm, outlet is divided into the six outlets and other portion is the same as that of the isomagnetophoretic immunoassay microchannel. The fabricated microchannel is shown in Fig. S1. The width of six

outlets was 30 μm and pitch between outlets was 24 μm. 10 mM Gd-DTPA solution was injected into the inlets 1 and 2 at 0.02 and 0.15 μL min^{-1}, respectively, and DI water was injected into the inlet 3 at 0.25 μL min^{-1}. The Gd-DTPA solutions at the respective positions were obtained from six outlets of an upper microchannel and the solution analyzed were 100 μL, respectively. The magnetic susceptibilities of a Gd-DTPA solution gradient obtained from respective outlets were measured by a superconducting quantum interference device (SQUID) magnetometer (Quantum Design MPMS–5T, Quantum Design, San Diego, CA). Using SQUID, the Gd-DTPA solutions were measured by applying magnetic field from −3 T to 3 T at intervals of 2,000–5,000 G at the 3×10^{-3} torr or lower pressure and 300 K for 80 min per solution. Since the standard holder for the magnetometer consists of a gelatin capsule designed for powder and dried samples, they melt if the solution is placed on the holder. Thus, we used a glass tube as a holder instead of the gelatin capsule. For obtaining the accurate results, the magnetic susceptibilities of respective solutions were corrected by subtracting the magnetic susceptibilities of the respective glass tubes from those of Gd-DTPA solutions within glass tubes. In addition, the glass tube was sealed with Parafilm to keep Gd-DTPA solutions from evaporation.

The resulting hysteresis loops of Gd-DTPA solutions are shown in Fig. S2a, where the slopes of the respective graphs represent the magnetic susceptibilities of Gd-DTPA solutions at the respective outlets. Because the Gd-DTPA solution from the sixth outlet corresponds to the transient region for magnetic susceptibility gradient, it was excluded from analyzing the linearity of magnetic susceptibility gradient. Accordingly, the magnetic susceptibility gradient of the Gd-DTPA solution in the upper microchannel was confirmed to be linear at the region above about 69 μm from the bottom of the microchannel, with values of -6.63×10^{-6} to -7.19×10^{-6} (Fig. S2b). From the measurement of Gd-DTPA solution gradient, the relation between χ and position (x) in a microchannel was induced to $\chi = 2.6546 \times 10^{-9}x - 7.35277 \times 10^{-6}$, where position ($x$) is a distance from the bottom of a microchannel.

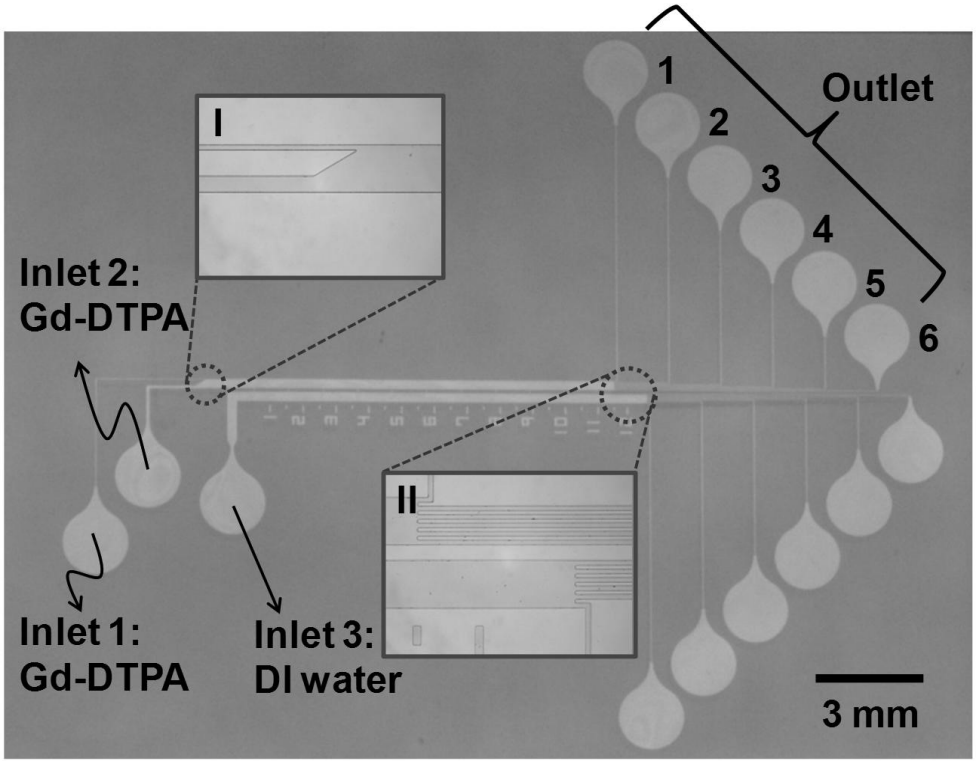

Fig. S1. A microchannel for confirming the Gd-DTPA solution gradient in an isomagnetophoretic immunoassay microchannel. The other region except for six outlets was the same as that of the isomagnetophoretic immunoassay microchannel.

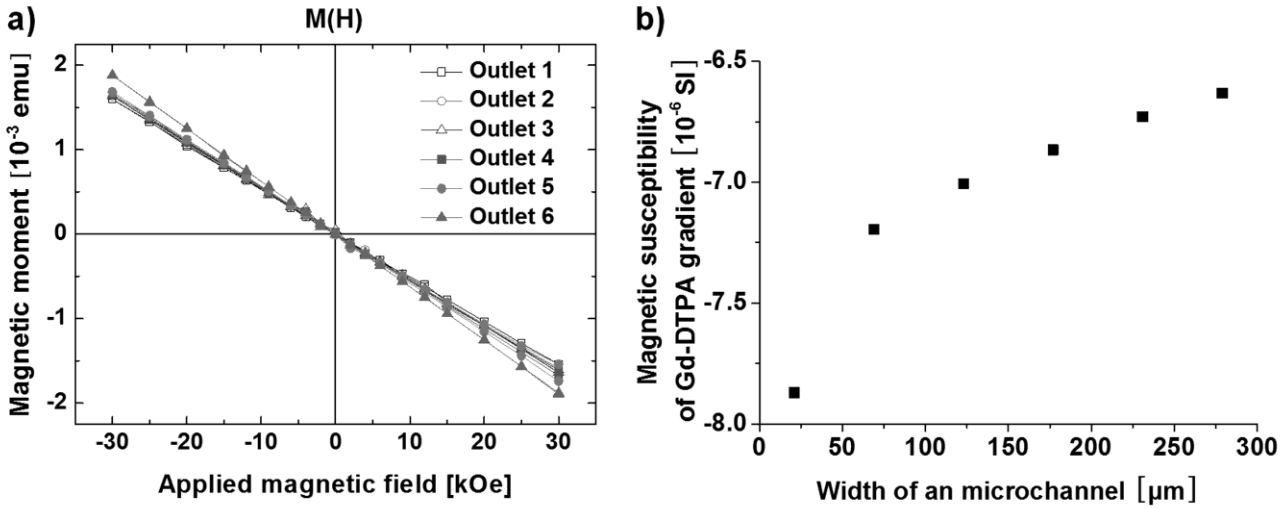

Fig. S2. The magnetic susceptibility of a Gd-DTPA solution gradient in an isomagnetophoretic immunoassay microchannel. (a) Hysteresis loops of the Gd-DTPA solution gradient in an isomagnetophoretic immunoassay microchannel, as measured by SQUID. (b) Magnetic susceptibilities of a Gd-DTPA solution at the respective positions in the upper microchannel.

Preparation of microbeads for two types of isomagnetophoretic immunoassays.

Anti-rabbit IgGs and three types of anti-breast cancer biomarkers (ER, PR and HER2) were conjugated to the respective microbeads using a general conjugation chemistry involving 1-ethyl-3-(3-dimethylaminopropyl)cabodiimide (EDC) plus sulfo-N-hydroxysuccinimide (sulfo-NHS). First, 200 μL of 10 μm microbeads (9.1×10^6 microbeads 200 μL^{-1}) and 10 μL of 6 μm fluorescent microbeads (2.1×10^6 microbeads 10 μL^{-1}) were prepared and washed by centrifugation with DI water at 13,200 rpm for 5 min three times. Finally, microbeads were resuspended in 300 μL of 0.1 M of 2-(N-morpholino)ethanesulfonic acid (MES) buffer (0.5 M NaCl, pH 6.0). Then microbeads were activated by adding 100 μL of 460 mM of sulfo-NHS and 100 μL of 156 mM of EDC sequentially and incubated for 30 min at room temperature. After incubation, the resulting microbeads were collected by centrifugation at 13,200 rpm for 5 min at 4 °C, and the supernatant was carefully discarded. The microbeads were resuspended in 50 μL of 0.15 M of phosphate-buffered saline (PBS) buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2). For isomagnetophoretic immunoassays of rabbit IgG-biotin, the activated 10 μm microbeads were incubated with 500 μL of 8 mg mL^{-1} of anti-rabbit IgGs for overnight in an orbital shaker at 150 rpm at room temperature. For isomagnetophoretic immunoassays of breast cancer biomarkers, the activated 6 μm yellow-green (YG), yellow-orange (YO) and bright blue (BB) fluorescent microbeads were incubated with 200 μL of 720 μg mL^{-1} anti-human ER, anti-human PR and anti-human HER2 at the same condition, respectively. The respective protein-conjugated microbeads were finally washed three times with 0.15 M of PBS buffer (1% BSA, 0.02% Tween, pH 7.2) and resuspended in 500 μL of the same buffer. The microbeads were kept at 4 °C until further use. After conjugation, the reacted microbeads were prepared for isomagnetophoretic immunoassays. The conjugated microbeads were counted by a hemacytometer (Marienfeld, Germany), and they were diluted into the reaction buffer solution (1X PBS, 0.02% Tween 20, pH 7.2). The concentration of resulting respective solutions for isomagnetophoretic immunoassays was prepared as followings: 2.32×10^7 microbeads mL^{-1} for rabbit IgG-biotin, 3.89×10^6 YG fluorescent microbeads mL^{-1} for ER, 3.75×10^6 YO fluorescent microbeads mL^{-1} for PR, 4.83×10^6 BB fluorescent microbeads mL^{-1} for HER2.

Procedure of isomagnetophoretic immunoassays of rabbit IgG-biotin

The number of microbeads conjugated with anti-rabbit IgGs for isomagnetophoretic immunoassays was adjusted to 16,240 microbeads in 40 μL of reaction buffer (1X PBS, 0.02% Tween 20, pH 7.2). 10 μL of rabbit IgG-biotin solution which was serially diluted in reaction buffer was added to the solution containing rabbit anti-IgG-conjugated microbeads. The resulting solutions were incubated for 10 min at room temperature. After incubation, 5 μL of the solution containing the MNPs conjugated with streptavidin was added to each mixture solution. Here, the volume and concentration of the solution of MNPs were fixed. The mixture solutions were mixed and incubated for another 10 min at room temperature. And then, the reaction buffer was exchanged using a microcentrifuge tube with 0.8 μm filter by centrifugation at 13,200 rpm for 1 min. The reaction buffer was exchanged for respective concentration of Gd-DTPA solution. The final mixture solutions were injected into the isomagnetophoretic immunoassay microchannel.

Breast cancer cell culture and lysate preparation

The human breast cancer cell lines (MCF-7; ATCC HTB-22, SK-BR-3; KCLB 30030) were selected for the isomagnetophoretic immunoassays of breast cancer biomarkers. Both of breast cancer cells were maintained in a T75 (Falcon flask) inside a humidified water-jacked incubator at 37 °C and 5% CO_2. MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin. SK-BR-3 cells were cultured in RPMI-1640 (RPMI-1640; Gibco, Grand Island, NY) containing 10% FBS and 1% penicillin/streptomycin. MCF-7 and SK-BR-3 cells were dissociated from the T75 (Falcon flask) using trypsin-EDTA solution (0.25 % trypsin and 1 mM EDTA·4Na) after rinsing the cells with PBS.

The dissociated MCF-7 and SK-BR-3 cells were diluted to a suitable concentration with two types of lysis buffers, respectively. Lysis buffer 1 (1 mM EDTA, 0.5 % Triton X-100, 5 mM sodium fluoride, 6 M urea, 1 mM activated sodium orthovanadate, 2.5 mM sodium pyrophosphate, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 100 μM PMSF, 3 μg/mL aprotinin in PBS, pH 7.3) was used for isomagnetophoretic immunoassays of ER and PR, and lysis buffer 2 (1% NP-40 alternative, 20 mM Tris, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM activated sodium orthovanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin) was utilized for isomagnetophoretic immunoassays of HER2. For MCF-7 and SK-BR-3 cell lysates, MCF-7 and SK-BR-3 cells were solubilized by lysis buffer 1 and 2 to be 6.05×10^6 cells mL^{-1} and sit on ice for 15 min. After two solutions were centrifuged at 2,000 g for 5 min, each supernate was prepared to be applied to the isomagnetophoretic immunoassays as follows. The supernate for isomagnetophoretic immunoassays of ER and PR was 6-fold diluted by diluent 1 (1 mM EDTA, 0.5% Triton X-100, 5 mM sodium fluoride in PBS, pH 7.3) and 2-fold diluted by diluent 2 (1 mM EDTA, 0.5 % Triton X-100, 5 mM sodium fluoride, 1 M urea in PBS, pH 7.3) and one for isomagnetophoretic immunoassays of HER2 was 12-fold diluted by diluent 3 (1% NP-40 alternative, 20 mM Tris, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM activated sodium orthovanadate).

Procedure of isomagnetophoretic immunoassays of breast cancer biomarkers

YG, YO and BB fluorescent microbeads conjugated with sheep anti-human ER, sheep anti-human PR and mouse anti-human HER2 for isomagnetophoretic immunoassays were adjusted to 11,670, 11,250 and 14,475 microbeads in 40 μL of reaction buffer (1X PBS, 1% BSA, 0.02% Tween 20, pH 7.2), respectively. Each 20 μL of ER, PR and HER2 standard solutions which were serially diluted in PBS were added to the respective solution containing sheep anti-human ER-conjugated microbeads, sheep anti-human PR-conjugated microbeads and mouse anti-human HER2-conjugated microbeads. The resulting solutions were incubated for 10 min at room temperature. After the first incubation, 7.2 μg mL^{-1} of biotinylated sheep anti-human ER, biotinylated sheep anti-human PR, and biotinylated goat anti-human HER2 solutions were added to the respective microbead solutions, followed by the second incubation for 10 min at room temperature. Finishing the second incubation, the solutions were changed with new 50 μL reaction buffer using a 0.8 μm filter by centrifugation at 13,200 rpm for 1 min to remove the biotinylated respective antibodies which were not reacted with microbeads. 5 μL solutions containing MNPs conjugated with anti-biotin were added to each mixture solutions. Here, the

volume and concentration of the solution of MNPs were fixed and all mixture solutions were finally incubated for 10 min at room temperature. And then, the solutions were exchanged with 25 mM Gd-DTPA solution using 0.8 μm filter by centrifugation at 13,200 rpm for 1 min for isomagnetophoretic immunoassays. The final respective solutions were injected into the isomagnetophoretic microchannel.

Analysis of magnetic susceptibility of microbead-MNP complexes using the number of MNPs estimated by the SEM analysis

For investigating the analytical resolution of this system, we performed a scanning electron microscope (SEM) analysis. Using the SEM analysis, we estimated the average number of magnetic nanoparticles bound on a microbead at the 19.8 aM concentration of rabbit IgG-biotin. To this end, we chose respective 10 microbeads according to the concentration of analyte and measured five selected surface regions such as middle, top, bottom, left and right regions in a microbead. After counting and averaging the number of MNPs on microbeads in these regions, the total number of MNPs was estimated by converting specific regions into the total area of a microbead. Fig. S3 shows the specific regions of microbeads in the respective cases. Before the SEM analysis of 19.8 aM analyte, we secured the SEM images of microbeads for control experiments (Fig. S3a). As can be seen in Fig. S3a, there are no MNPs on the microbead resulting from control experiments. However, there are about 53.1 MNPs per a microbead reacted with 19.8 aM analytes, statistically (n = 10, see Fig. S3b).

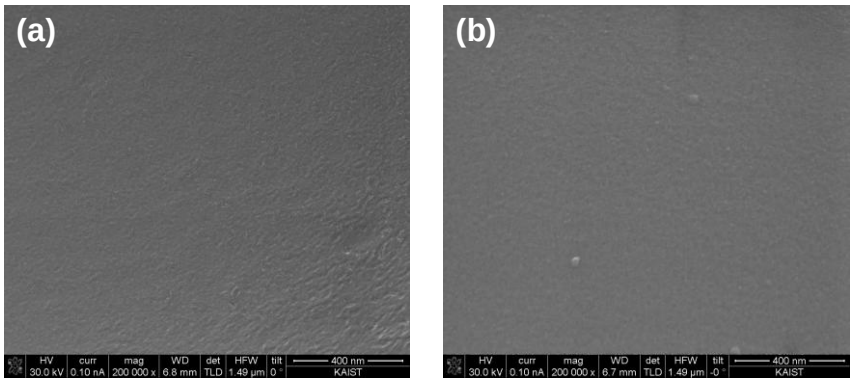

Fig. S3. SEM images of surface of microbeads: (a) control experiment, (b) a microbead reacted with 19.8 aM analyte (rabbit IgG-biotin).

In isomagnetophoresis, the magnetic susceptibilities of a material and surrounding solution are the same at the isomagnetophoretic point. On the basis of this principle, we can calculate the magnetic susceptibilities of microbead-MNP complexes in the respective cases using the number of MNPs estimated by the SEM analysis. A magnetic force exerting on a particle is zero under the isomagnetophoretic circumstance if a material is located on its iso-point. In other words, the repulsive magnetic force mainly induced by the polystyrene microbead whose magnetic susceptibility is negative and the attractive force induced by MNPs whose magnetic susceptibility are positive are the same and have opposite signs (Eq. S2, S3).

$$\vec{F}_{mag} = \Delta\chi_T V_T \nabla\left(\frac{B^2}{2\mu_0}\right) = \vec{F}_{repulsive} + \vec{F}_{attractive} = 0 \qquad \text{(S2)}$$

$$F_{repulsive} = -F_{attractive} \qquad \text{(S3)}$$

Here, Eq. S3 is summarized as Eq. S4 because B (magnetic field) and μ_0 (vacuum permeability) is the same between attractive and repulsive magnetic forces.

$$\Delta\chi_{PS} V_{PS} = -\Delta\chi_{MNP} V_{TMNP}, \qquad \text{(S4)}$$

where χ_{PS} is the magnetic susceptibility of a polystyrene microbead, χ_{MNP} is the magnetic susceptibility of a MNP, V_{PS} is the volume of a microbead, V_{TMNP} is the total volume of MNPs conjugated with a microbead according to the concentration of analytes and V_{MNP} is the volume of a MNP. Because Δ can be rewritten into the difference form and the total volume of MNPs is multiply of the number of MNPs conjugated and volume of a MNP, Eq. S4 is the same as Eq. S5.

$$(\chi_{PS} - \chi_S)V_{PS} = -(\chi_{MNP} - \chi_S)V_{TMNP} = -N(\chi_{MNP} - \chi_S)V_{MNP}, \qquad \text{(S5)}$$

where N is the total number of MNPs conjugated.
Finally, the magnetic susceptibility of microbead-MNP complex is presented as follows:

$$\chi_S = \frac{(\chi_{PS}V_{PS} + N\chi_{MNP}V_{MNP})}{NV_{MNP} + V_{PS}} \qquad \text{(S6)}$$

Using Eq. S6 and the SEM analysis, the magnetic susceptibilities of microbead-MNP complexes were calculated. For using Eq. S6, we used values of a microbead whose diameter and magnetic susceptibility were 10.269 ± 0.505 μm provided from the manufacturer and -8.21×10^{-6} [SI, volumetric] provided from the literature[9] and the values of MNPs whose diameter and magnetic susceptibility were 67.2 ± 4.5 nm and 0.0014 [SI, volumetric] from the reference 10. This diameter value was almost similar with the value measured by the SEM image, 63.2 ± 8.5 nm. With these values and the number of MNPs bound on the microbeads, the magnetic susceptibilities of microbeads reacted with 19.8 aM analytes were calculated. As mentioned above, the number of MNPs per a microbead at 19.8 aM analytes was revealed to 53.1. Based on this result, their magnetic susceptibility calculated by the SEM analysis was -8.00×10^{-6}. Meanwhile the magnetic susceptibility of microbeads in control experiment was -8.21×10^{-6}, which is the same with that of polystyrene microbead itself. Thus, isomagnetophoretic immunoassay can discriminate the 0.21×10^{-6} difference in magnetic susceptibility so that this method turned out to be very sensitive to detect attomolar concentrations of analytes.

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