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Novel detection system for biomolecules using nano-sized bacterial magnetic particles and magnetic force microscopy

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Abstract

A system for streptavidin detection using biotin conjugated to nano-sized bacterial magnetic particles (BMPs) has been developed. BMPs, isolated from magnetic bacteria, were used as magnetic markers for magnetic force microscopy (MFM) imaging. The magnetic signal was obtained from a single particle using MFM without application of an external magnetic field. The number of biotin conjugated BMPs (biotin-BMPs) bound to streptavidin immobilized on the glass slides increased with streptavidin concentrations up to 100 pg/ml. The minimum streptavidin detection limit using this technique is 1 pg/ml, which is 100 times more sensitive than a conventional fluorescent detection system. This is the first report using single domain nano-sized magnetic particles as magnetic markers for biosensing. This assay system can be used for immunoassay and DNA detection with high sensitivities.

Keywords: Bacterial magnetic particles; Magnetic force microscopy; Magnetosensor; Streptavidin-biotin interaction

1. Introduction

Magnetic particles are useful magneto-sensor markers for quantitative detection of molecular interactions, including those between antigen-antibody, DNA–DNA and ligand-receptor. Measurements are traditionally performed using superconducting quantum interference device (SQUID) magnetometer (Enpuku et al., 1999, 2001), giant magneto-resistive (GMR) sensors (Baselt et al., 1998; Edelstein et al., 2000) or by measurement of magnetic permeability (Kriz et al., 1996, 1998). These types of magneto-sensors are highly sensitive and theoretically can detect single magnetic particles (Edelstein et al., 2000). Magnetic

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force microscopy (MFM) is commonly used for high resolution magnetic imaging (Grütter et al., 1992) and can be useful for the detection for molecular interactions.

Magnetic bacteria contain a single internal chain of magnetite particles, which run the long axis of the bacterial body. Intracellular magnetite produced by magnetic bacteria (Balkwill et al., 1980), have uniform size and shape when compared with artificial magnetite. These bacterial magnetic particles (BMPs) are single crystals having a single magnetic domain (Towe and Moench, 1981). The magnetite is nano size (50–100 nm) and covered with a lipid bilayer membrane mainly of phosphatidylethanolamine (Nakamura et al., 1991). BMPs are ferrimagnetic, however have excellent dispersion in aqueous solutions imparted by the lipid membrane (Nakamura and Matsunaga, 1993). The phosphatidylethanolamine can be used for immobilization of biomolecules such as antibodies, enzymes and DNA by targeting the amine group with specific cross-linking reagents (Matsunaga et al., 1996; Tanaka and Matsunaga, 2000; Nakayama et al., 2003).

In this study, we focused on the utilization of BMPs as magnetic markers for biosensing. Intracellular chains of BMPs were previously detected utilizing MFM without the application of an external magnetic field (Proksch et al., 1995; Suzuki et al., 1998). Here we performed MFM imaging of single particles to evaluate applicability of BMPs as magnetic markers without introduction of an induced magnetic field, simplifying the process.

2. Materials and methods

2.1. Materials

Amine coated glass slides were purchased from Pax Genetica (Seoul, Korea). Sulfosuccinimidy1-6′-(biotinamido)-6-hexanamido hexanoate (Sulfo-NHS-LC-LC-Biotin) was purchased from Pierce (Rockford, IL, USA). Polyethylene glycol (PEG) derivative, Biotin-PEG-NHS (M_W = 3400) was purchased from Shearwater Corp. (Huntsville, AL, USA). Streptavidin was purchased from New England BioLabs Inc. (Beverly, MA, USA). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). Other reagents were commercially available analytical reagents or laboratory grade materials.

2.2. Preparation of magnetic markers, biotin conjugated to bacterial magnetic particles

BMPs were prepared from *Magnetospirillum magneticum* AMB-1 according to a previously described method (Tanaka and Matsunaga, 2001). Biotin conjugated to BMPs (biotin-BMPs) was prepared using Sulfo-NHS-LC-LC-Biotin or Biotin-PEG-NHS containing *N*-hydroxysuccinimidyl esters. A 1 mg/ml BMPs suspension in 10 mM phosphate buffered saline (PBS: pH 7.3) containing 5 mg/ml Sulfo-NHS-LC-LC-Biotin or Biotin-PEG-NHS solution was prepared. The suspension was dispersed well by pulse sonication for 30 min at room temperature. The modified BMPs were magnetically separated from the reaction mixture using a neodymium boron (Nd-B) magnet, and washed three times with 1 ml of PBS to remove excess Sulfo-NHS-LC-LC-Biotin or Biotin-PEG-NHS.

2.3. Imaging of single bacterial magnetic particles using MFM

BMPs were suspended in distilled water and the concentration was adjusted to 10 μg/ml. The suspension was used to cover the glass slides and left to dry in air for 1 h. The glass slides were gently washed with distilled water and air dried. BMPs on the glass slides were imaged using a Seiko Instruments SPI 3800 magnetic force microscope (Seiko Instruments Inc., Tokyo, Japan). MFM imaging using phase detection was performed in lift mode at a 50 nm distance between the cantilever tip and the surface of the glass slide. The 50 nm distance was determined after topography was measured in non-contact mode. Atomic force microscopy (AFM) and MFM imaging was performed at a scan rate of 0.5 Hz, with a resolution of 256 pixels/line. The MFM cantilevers were made of silicon coated with cobalt-chromium alloy (Hc = 300–400 Oe, spring constant: 1–5 N/m, MFMR-10, Nanosensors Inc., Darmstadt, Germany) or cobalt-platinum-chromium alloy (Hc = 650 Oe, spring constant: 20 N/m, Seiko Instruments Inc., Tokyo, Japan). The cantilever with coercive force of 300–400 Oe was used for MFM imaging in air, and the cantilever with coercive force of 650 Oe was used for MFM imaging in a vacuum.

2.4. Streptavidin immobilization on a glass slide

Biotin was immobilized by applying 10 mg/ml Sulfo-NHS-LC-LC-Biotin or 20 mg/ml Biotin-PEG-NHS to amine treated glass slides. The glass slides were incubated for 2 h at room temperature then gently washed twice with distilled water. A microarray spotter (SPBIO, Hitachi Software, Yokohama, Japan) was used to apply a 600 μg/ml streptavidin solution containing 40% (v/v) dimethyl sulfoxide (DMSO). The glass slides were incubated for 1 h at room temperature then washed twice with PBS containing 0.25% SDS (PBS-SDS) and twice with distilled water.

2.5. Magnetic sensing procedure

Illustration of the biotin-BMPs immobilization process for magnetic sensing on streptavidin modified glass slides is shown in Fig. 1. Biotin-BMPs bound to the modified glass slides were observed by differential interference microscopy (IX 70, Olympus, Tokyo, Japan). The glass slides were incubated with 100 μg/ml BSA to prevent biotin-BMPs from nonspecific binding, then washed twice with PBS-SDS and twice using distilled water. Then, 25 μl of a 1 mg/ml Biotin-BMPs suspension was put on the streptavidin modified glass slides and incubated for 1 h at room temperature. The glass slides were washed twice with PBS-SDS, twice using distilled water and dried in air. The glass slides were then observed by differential interference microscopy. The glass slides used for magnetic detection using MFM was performed using the described method, but used different streptavidin (0.001–100 pg/ml) and biotin-BMPs concentrations (100 μg/ml). AFM and MFM imaging was performed using the 300–400 Oe cantilever in air. The frequency of biotin-BMPs bound to the streptavidin on the glass slides was determined by direct magnetic signal counts from the magnetic field of BMPs using MFM.

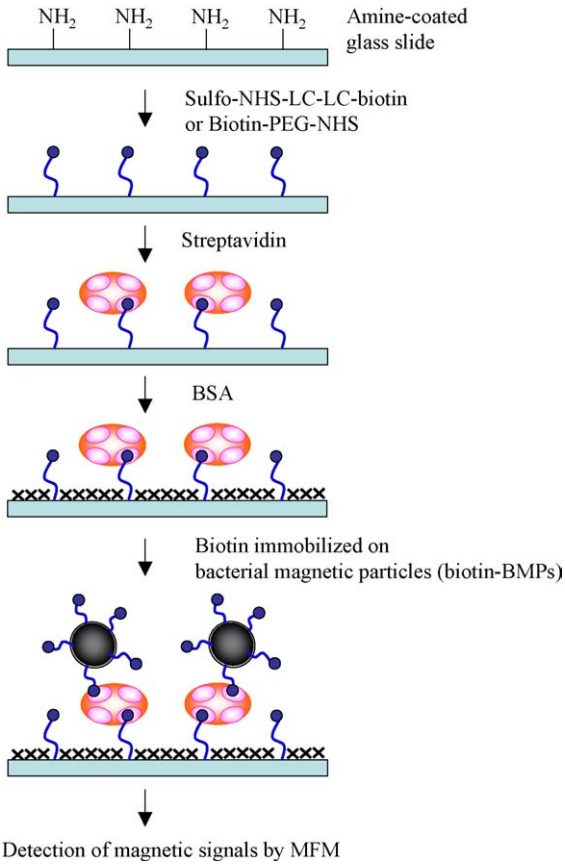

Fig. 1. Procedure of magnetic detection for streptavidin by using biotin-BMPs and MFM.

3. Results and discussion

3.1. Evaluation of magnetic markers by MFM

Typical AFM and MFM images of BMPs using the standard MFM cantilever (Hc = 300–400 Oe) are shown in Fig. 2A and B. The AFM images indicate sufficient resolution for single BMPs. Repulsive and attractive interactions between the tip and sample are represented by bright (repulsive) and dark (attractive) contrast. During MFM imaging bright and dark contrasts are observed depending upon the magnetic polarity. The MFM image from BMPs should generate both bright and dark images dependent upon which magnetic pole is detected. However, only dark images were observed with BMPs using the 300–400 Oe cantilever (Fig. 2B). Use of the cantilever with higher coercive force (Hc = 650 Oe) under vacuum did however provide contrast indicating the magnetic polarity of BMPs (Fig. 3B). Attractive interactions detected between the standard cantilever tip and BMPs might be due to relatively low coercive force (Hc = 300–400 Oe) and

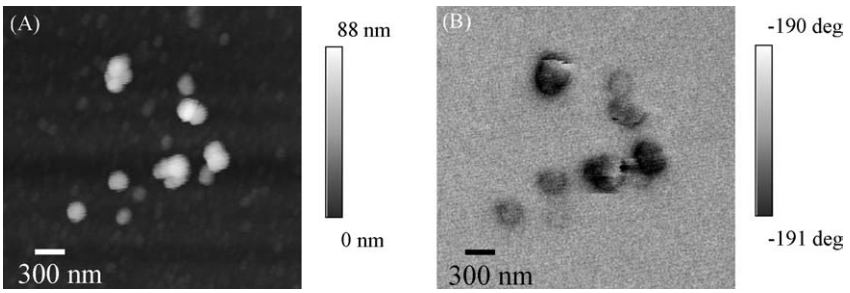

Fig. 2. AFM (A) and MFM (B) images of BMPs on the glass slide. MFM imaging was performed by using the MFM cantilever with 300–400 Oe in air.

subsequent magnetization of the cantilever by BMPs. Although the contrast between magnetic poles could not be determined in air, uniform magnetic signals from a single BMP were obtained using a standard cantilever (Fig. 2B), indicating the utility of this method for biosensing. In the following experiments, the standard MFM cantilever was used.

3.2. Biotin-BMPs bound to streptavidin immobilized on a glass slide

A differential interference microscopic image of biotin-BMPs bound to a glass slide is shown in Fig. 4A. Biotin-BMPs immobilized on modified slides were clearly observed on the spots of streptavidin (approximately 200 μm in diameter). Binding was clearly observed only when Biotin-PEG-NHS (Mw = 3400, spacer arm: 340 Å) was used as the crosslinking reagent for both the glass slide and BMPs modification. An image was generated using Sulfo-NHS-LC-LC-Biotin (Mw = 669.75, spacer arm: 30.5 Å) however binding was observed only at the fringe of streptavidin spots (Fig. 4C). This phenomenon results from to short a spacer arm length and possible interferences between the biotin-BMPs binding due to the close proximity of reactive streptavidin sites. Furthermore, the surface of the glass slide and BMPs treated with Biotin-PEG-NHS are expected to be more hydrophilic than those treated with Sulfo-NHS-LC-LC-Biotin, because the lower contact angle on the glass slide after treatment with Biotin-PEG-NHS was confirmed by dropping the water on the glass slides. The higher hydrophilicity of the glass slide may allow higher reactivity of biotin-BMPs to streptavidin on the glass slide. No binding was observed when untreated BMPs were used (Fig. 4B and D).

3.3. Streptavidin detection using MFM

Magnetic detection of streptavidin on modified glass slides using biotin-BMPs was performed by direct BMP counts using MFM. Biotin-BMPs were

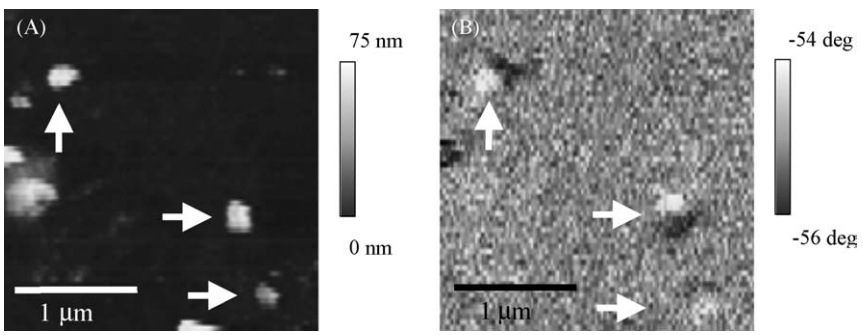

Fig. 3. AFM (A) and MFM (B) images of BMPs using the high coercivity cantilever (650 Oe) in a vacuum.

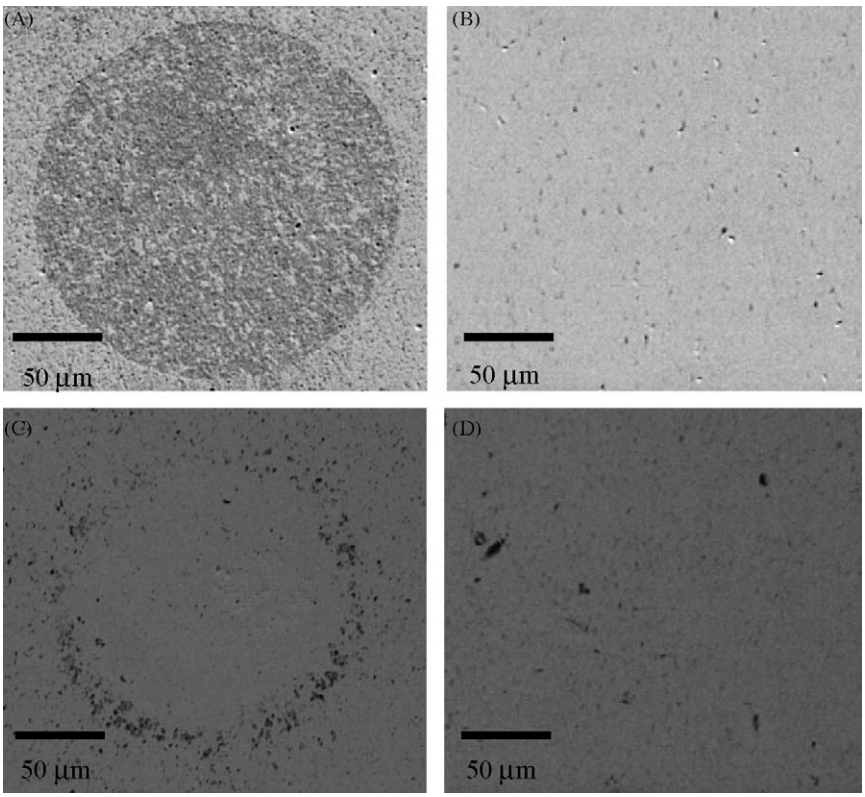

Fig. 4. Differential interference microscopic photographs of streptavidin immobilized on the glass slides after addition of biotin-BMPs (A and C) and non-treated BMPs (B and D). Biotin-PEG-NHS (A and B) and Sulfo-NHS-LC-LC-Biotin (C and D) were used as cross-linking reagents for immobilization of biotin onto BMPs and the glass slides.

applied to biotin immobilized on the glass slide after treatment with various concentrations of streptavidin (0.001–100 pg/ml). The biotin-BMPs concentration was optimized at 100 μg/ml. More than 200 μg/ml of biotin-BMPs promotes aggregation in aqueous solution and highly nonspecific attachments. In the MFM images, immobilized BMPs are small dark areas on the contrasting background. The relationship between streptavidin concentrations and BMP numbers in a 20 μm × 20 μm area are shown in Fig. 5. Increased biotin-BMP numbers occurred up to 100 pg/ml streptavidin. Concentrations of streptavidin greater than 100 pg/ml results in aggregates of biotin-BMPs on the glass slide made precise counting impossible (more than 1000 BMPs/20 μm × 20 μm). Biotin-BMP binding difference was not observed in the absence and up to 0.1 pg/ml streptavidin concentration. The minimum detection limit for streptavidin

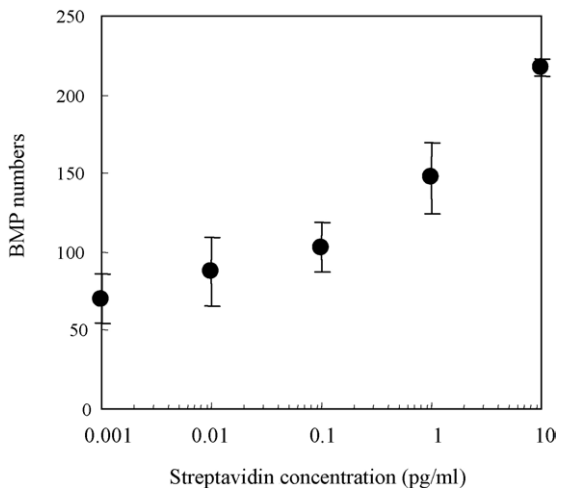

Fig. 5. Relationship between streptavidin concentration and BMP numbers at 20 μm × 20 μm area when streptavidin (0.001–10 pg/ml) was applied to biotin immobilized on the glass slide.

was 1 pg/ml. For a comparison, a fluorescent detection of Cy3-labeled streptavidin binding onto a biotinylated glass slide was performed by a photomultiplier using a ScanArray fluorescent scanner (GSI Lumonics Inc., Ontario, Canada). The photomultiplier has a potential to detect 40 molecules of Cy3 or Cy5 in a 20 μm × 20 μm. The minimum detection limit of Cy3-streptavidin was 100 pg/ml of streptavidin, which corresponds to approximately 2000 molecules of streptavidin in the same area if all streptavidin molecules were immobilized on their surface. Fluorescent signals at less than 10 pg/ml streptavidin was not able to discriminate from background noise. This detection limit is almost the same with a previous report (150 pg/ml of IgG) by using a laser scanning system with a photomultiplier (Axon) (Wacker et al., 2004). Therefore, we concluded that the experimental results by BMPs-based assay are 100 times more sensitive for the detection of streptavidin compared with fluorescent detection, suggesting its use has potential advantages for extremely sensitive biomolecule detection.

In recent studies, micro size magnetic particles or aggregates of nano size magnetic particles have been used as magnetosensors. The detection of a single magnetic probe using small magneto-resistive spin valve sensor has been reported. The size of magnetic particle used was however micro size (2 μm) and the detection range was narrow, 6–20 particles were needed for magnetic signal detection (Graham et al., 2002). These particles are too large for use as markers in place of fluorescent dyes or luminescent reagents.

In this study, we developed a high sensitivity detection system for streptavidin using nano-sized single domain magnetic particles and MFM. This is the first report detecting magnetic signal from a single nano size magnetic marker for biosensing. Biotinylated DNA or antibody can also be immobilized on BMPs by using Biotin-PEG-NHS and streptavidin. The functionalized BMPs can be useful for detection of hybridization or immunoassay on the solid support, thus this assay system will be applied to highly sensitive DNA detection and immunoassay.

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