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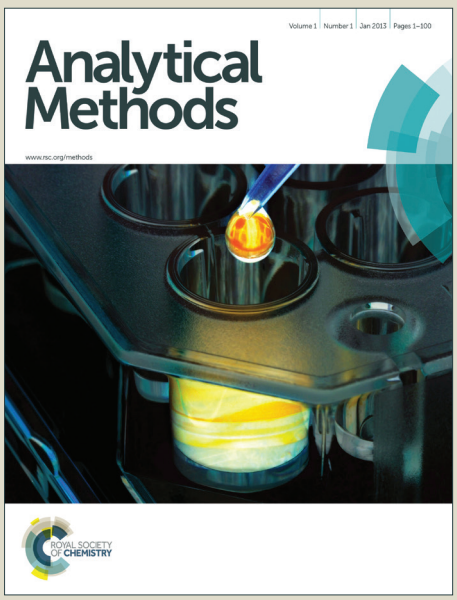

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Magnetic Immunoassay Based on Frequency Mixing Magnetic Detection and Magnetic Particles of Different Magnetic Properties

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Abstract

This paper presents a novel analytical system that employs two different types of magnetic particles (MP) with Frequency mixing magnetic detection (FMMD). Model experiment with MP and FMMD show that this method significantly reduces the steps involved in common immunobiological assays and enables repetition of the measurement without signal decay.

Main Manuscript

Magnetic particles (MP) have been widely used in many fields including biochemistry and medicine[1]. The most common usage of MP in biochemistry is as a capturing agent for isolation and enrichment of the analyte(s) from complicated samples because of the easy magnetic manipulation[2, 3]. Other biomedical applications include their use as image tracers and contrast agents for Magnetic Resonance Imaging (MRI) and Magnetic Particle Imaging (MPI) and for drug delivery to specific targets[4-8]. Recently, the capability of MP as markers for labelling bio-materials such as DNA, proteins and even cells in a bioassay have been extensively researched and have led to many commercial applications[1, 9]. As markers in bioassays, MP is relatively stable because there is no signal decay, resulting in less fluctuation in signal intensity than those frequently observed in fluorescence- and enzyme-based assays. Among the various MP used, superparamagnetic nanoparticles are widely employed as markers because of their small disturbance to biochemical processes and because of their favourable magnetic properties[10].

The use of magnetic particles for both manipulation of a sample and for labelling the analyte is beneficial because it simplifies the assay procedure. However, the following issues should be considered when using MP for both capturing and labelling the sample in one reaction simultaneously. The most important one is that the Probe Magnetic particles (PMP) generating the signal should not be separable by the usual magnets for magnetic separation. The PMP

should be attached to the capturing agent through a biochemical reaction with the analyte. Only the capturing agent should be captured by the separation magnet. Finally, the magnetic reader should detect a magnetic signal only from the PMP, with a high selectivity to PMP as compared to other materials.

To fulfil these considerations, magnetic nanoparticles with 20 nm diameter comprising a single magnetic domain were employed as the PMP. 500 nm diameter magnetic particles with multiple magnetic domains which can be attracted by the external magnetic field were used as the Capturing Magnetic Particles (CMP). The PMP could not be separated by a standard separation magnet because their induced magnetic moment is too small due to their small size. Thus, the PMP could only be separated when they were attached to the CMP. Only the large CMP (with more than 50 nm diameter) exhibit a sufficiently large magnetic moment to be attracted by the external magnet. For the measurement and quantification, the relatively new Frequency Mixing Magnetic Detection (FMMD) technique was used. Previous research showed the applicability of this method for the detection of PMP[11]. Meyer *et al.* showed that C-reactive protein, *Yersinia pestis* and *Francisella tularensis* labelled with PMP can be assessed by FMMD[12-14]. Hong *et al.* used the method by combining enzyme-linked immunofiltration assay and FMMD, resulting in the treatment of multiple samples and eliminating a few of the steps required for usual ELISA assays[15]. By discriminating the specific nonlinear signatures of different types of magnetic nanoparticles by observation of the frequency mixing components of higher order, it has been shown that two different types of MP can be individually and simultaneously detected[16].

To the best of our knowledge, this is the first bioassay employing two different magnetic beads together for separation and labelling in one analytical procedure. Furthermore, the FMMD technique can assess the amount of superparamagnetic material in the sample resulting in the direct measurement of the sample without the need of transferring the sample. Repeated measurements of the sample can easily be done. This is not possible for the usual immunobiological assays employing enzymatic or fluorescent markers as labelling compounds because of rapid degeneration.

FMMD consists of a measurement head and an electronics for excitation and detection frequency generation and for synchronous readout of the measured sum frequency component[11,17]. The magnetic measurement head comprises two excitation coils wound on the same bobbin and the pick-up coil with two identical coils placed next to each other but wound in opposite direction. The outer low frequency coil generates a 4 mT magnetic field at 61 Hz which periodically drives the magnetic particles close to saturation. The other coil generates an excitation field of 0.5 mT at 61 kHz. The magnetic moment sensitivity is given by the Johnson noise of the detection coil. Based on the previous research[11], the noise equivalent magnetic moment was calculated to 1.0×10^{-13} $Am^2/\sqrt{Hz}$. In this method, two distinct frequencies are applied to the superparamagnetic particles, resulting in the appearance of harmonic peaks and of

intermodulation products, due to the nonlinear magnetic characteristics of the MP. These peaks are highly specific to the nonlinearity of the magnetic particles.

For the model experiments, two different types of magnetic particles were employed. PMP with a hydrodynamic diameter of 20 nm (Nanomag®-D-spio-biotin) were purchased from Micromod Patrikletechnologie GmbH (Rostock, Germany). According to the manufacturers description, the particles consist of about 55~85% (W/W) iron oxide (magnetite) in a matrix of dextran (MW:40,000 Da), with varying diameter and surface modification. Electron microscope data showed that 20 nm particles contain iron oxide crystals of 7.0~10 nm[18]. The PMP were diluted 100-fold in Phosphate Buffered Saline (PBS, pH 7.5). Particles with a hydrodynamic diameter of 500 nm from the same manufacturer, covered with biotin and also diluted 100-fold in PBS, were employed as the CMP. The 500 nm particles have a core of magnetite and a dextran shell. Based on the manufacturer's description, they can be separated with a conventional permanent magnet. Avidin (Sigma Aldrich, MO) was serially diluted in PBS containing 1.0% of bovine serum albumin (0.0, 3.0, 6.0, 12.0, 24.0, and 48.0 mg/mL), and 900 μL of the serially diluted Avidin solution was mixed with 100 μL of the CMP. The mixture was incubated for 15 min at room temperature. The mixture was then washed three times and re-dissolved in 1.0 ml of PBS. Subsequently, 100 μL was transferred to another tube and the amount of PMP was measured to determine the value of the unbound CMP. Then, 100 μL of the PMP solution was added and incubated for another 15 min. Finally, the mixture was washed again with PBS three times and re-dissolved in 1.0 mL of PBS. From this, 100 μL was measured with FMMD and the final value was obtained by subtracting the CMP measured above. The whole experiment was triplicated. For statistical evaluation, a two-tailed Student t-test was performed using Minitab 16 (Minitab Inc., PA) with a cut-off of $p<0.05$.

Scheme 1 provides a schematic drawing of the analytical procedures used in this research. At the first step, the CMP separated the Avidin analyte from the sample. Subsequently, the PMP were attached to the CMP by the interaction between Avidin and its ligand, biotin. Thus, the amount of bound PMP is proportional to the amount of analyte. Next, after magnetic separation, unbound PMP are washed out. Only the PMP bound to CMP are quantified by the magnetic reader. In this work, the discrimination between PMP and CMP is performed by recording the difference of the two signals before and after magnetic separation. This analytical technique does not require different chemical procedures and transfer of the sample between the procedures because all the materials for the separation and labelling material are magnetic particles. This leads to a reduction of practical errors during the assay. In addition, the effect of deterioration with time can be neglected because the magnetic properties of the magnetic particles do not change with time. However, in case of other

bioassays employing enzymes or fluorescence, timing is a critical factor for the results because of the dynamic and unstable properties of the substances used.

[Position for Schematic 1]

Figure 1 shows the results from the model experiment based on the principle suggested in Scheme 1. Figure 1(a) shows the differences of nonlinear magnetization of the 20 nm and the 500 nm MP which are the basis of the experiments. The 20 nm magnetic beads cannot be separated by the permanent magnet while the 500 nm magnetic beads can easily be retained by the magnet. This means that 20 nm PMP can only be separated when they are bio-chemically attached to the 500 nm CMP. Figure 1(b) shows that unspecifically bound PMP without Avidin linkage to CMP do not have a significant effect on the intensity of the signal. Instead, the measurement of bound PMP shows that addition of Avidin significantly increases the signal intensity, meaning the signal intensity is related to the amount of Avidin analyte in the solution.

[Position for Figure 1]

Figure 2 shows that the increase in Avidin concentration corresponds to the amount of the PMP used in the experiment. By performing a linear fit to the data using Origin 8.5, a linear dependence ranging from 9.39 to 37.9 ug/ml was found, with slope=2.17 and intercept=16. The coefficient of determination (R^2) for the linear approximation was 0.96. The linear fit yielded an offset of the signal intensity of 500 nm for zero PMP concentration. The results from the measurement performed 3 hours after the first experiment showed that there was no significant change. It is impossible to obtain such a result for enzyme- or fluorophore-based immunoassays.

[Position for Figure 2]

Although magnetic beads have shown great utility in diagnostics and immunoassays both as separation agents and as markers, little progress has been made on the commercialization and standardization of the whole analytical system. We think that this is due to the lack of proper analytical procedures after manipulation of the analyte by MP. Normally, the analyte is labelled by a fluorophore or enzyme, instead of magnetic particles, after the magnetic separation[10]. However, there are some drawbacks to using these conventional chemical and biochemical markers because proper quantification cannot be performed due to light scattering, aggregation, and distortion of signals from MP stacking. Magnetic particles have not been used as markers because they would separate together. Furthermore, no suitable

magnetic reader has been available that could selectively retrieve information on the magnetic signals from the PMP which are free in the solution. To date, magnetic field sensors such as Giant magnetoresistance(GMR) and Superconducting Quantum Interference Device (SQUID) have shown outstanding performance on the detection of PMP[19-21]. However, in order to use GMR or spin valve sensors, the PMP must be immobilized on the surface, which requires the transfer of the sample and additional chemical treatment. In addition, these sensors have a small surface area, are laborious to manufacture, and have a very narrow detection range. For example, the maximum number of PMPs of 50 nm size occupying an area of 100 μm × 100 μm is about 4 million. SQUID suffer from similar problems, in addition to their cooling requirements, a very expensive operation cost and the necessity for a skilled operator. [10, 22]. FMMD is based on the frequency mixing at the non-linear magnetization curve of superparamagnets. When the sample is excited by the two distinct frequencies, the response signals of a linear combination ($m \cdot f_1 + n \cdot f_2$) is measured[11]. The signal is representing the total amount of particles in a certain volume, avoiding the immobilization of the biomolecules on two- or three-dimensional substrates.

Conclusions

In summary, a new bio analytical method employing two different MP and a FMMD reader has been established. The main idea of this study is to make use of the different magnetic properties of MP and the selectivity of the FMMD technique. The large particles are used for magnetic separation, the small particles for magnetic quantification by comparing the magnetic signal before and after separation. Our results from the model experiments employing biotin and Avidin showed that this method eliminates many of the typical procedures required by conventional photometric methods. Additional benefits are that the operator does not have to continue the labelling, quench the reaction, and perform measurements in time, which are critical in other assays. Aggregation, stacking, and scattering of light are not an issue in this study because FMMD techniques measure the sum of the signals of all the MP in the sample. This method can increase the effectiveness and potential capability of bioassays based on magnetic particles. With the scheme presented here, the field of potential applications can be extended to most of the bioassays.

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Notes and references

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References

1. R. Ramanujan, in *Biomedical Materials*, ed. R. Narayan, Springer US, 2009, pp. 477-491.
2. S. Berensmeier, *Appl Microbiol Biotechnol*, 2006, **73**, 495-504.
3. M. Franzreb, M. Siemann-Herzberg, T. Hobley and O. T. Thomas, *Appl Microbiol Biotechnol*, 2006, **70**, 505-516.
4. S. R. Dave and X. Gao, *Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology*, 2009, **1**, 583-609.
5. L. Josephson, in *BioMEMS and Biomedical Nanotechnology*, eds. M. Ferrari, A. Lee and L. J. Lee, Springer US, 2006, pp. 227-237.
6. J. S. Weinstein, C. G. Varallyay, E. Dosa, S. Gahramanov, B. Hamilton, W. D. Rooney, L. L. Muldoon and E. A. Neuwelt, *J Cereb Blood Flow Metab*, 2009, **30**, 15-35.
7. B. Gleich and J. Weizenecker, *Nature*, 2005, **435**, 1214-1217.
8. P. W. Goodwill, E. U. Saritas, L. R. Croft, T. N. Kim, K. M. Krishnan, D. V. Schaffer and S. M. Conolly, *Adv Mater*, 2012, **24**, 3870-3877.
9. E. Choi, D. Loo, J. W. Dennis, C. A. O'Leary and M. M. Hill, *Electrophoresis*, 2011, **32**, 3564-3575.
10. V. Mani, B. V. Chikkaveeraiah and J. F. Rusling, *Expert opinion on medical diagnostics*, 2011, **5**, 381-391.
11. H.-J. Krause, N. Wolters, Y. Zhang, A. Offenhäusser, P. Miethe, M. H. F. Meyer, M. Hartmann and M. Keusgen, *Journal of Magnetism and Magnetic Materials*, 2007, **311**, 436-444.
12. M. H. Meyer, M. Hartmann, H. J. Krause, G. Blankenstein, B. Mueller-Chorus, J. Oster, P. Miethe and M. Keusgen, *Biosens Bioelectron*, 2007, **22**, 973-979.
13. M. H. Meyer, M. Stehr, S. Bhuju, H. J. Krause, M. Hartmann, P. Miethe, M. Singh and M. Keusgen, *Journal of microbiological methods*, 2007, **68**, 218-224.
14. M. H. F. Meyer, H.-J. Krause, M. Hartmann, P. Miethe, J. Oster and M. Keusgen, *Journal of Magnetism and Magnetic Materials*, 2007, **311**, 259-263.
15. H. B. Hong, H. J. Krause, K. B. Song, C. J. Choi, M. A. Chung, S. W. Son and A. Offenhausser, *J Immunol Methods*, 2011, **365**, 95-100.
16. L. Lenglet, *Journal of Magnetism and Magnetic Materials*, 2009, **321**, 1639-1643.
17. H. Hong, H. J. Krause, S. Sohn, T. Baik, J. H. Park, S. Shin, C. Park and D. Song, *Anal Biochem*, 2014, **447**, 141-145.
18. S. N. Tabatabaei, J. Lapointe and S. Martel, Intelligent Robots and Systems, 2009. IROS 2009. IEEE/RSJ International Conference on, 2009.
19. D. Mitra, Ramli, H. Freddy and Khairurrijal, *GMR Biosensors for Clinical Diagnostics*, 2011.
20. L. Hao, D. Cox, P. See, J. Gallop and O. Kazakova, *Journal of Physics D: Applied Physics*, 2010, **43**, 474004.
21. Y. R. Chemla, H. L. Grossman, Y. Poon, R. McDermott, R. Stevens, M. D. Alper and J. Clarke, *Proceedings of the National Academy of Sciences of the United States of America*, 2000, **97**, 14268-14272.
22. C. S. Thaxton, H. D. Hill, D. G. Georganopoulou, S. I. Stoeva and C. A. Mirkin, *Analytical chemistry*, 2005, **77**, 8174-8178.

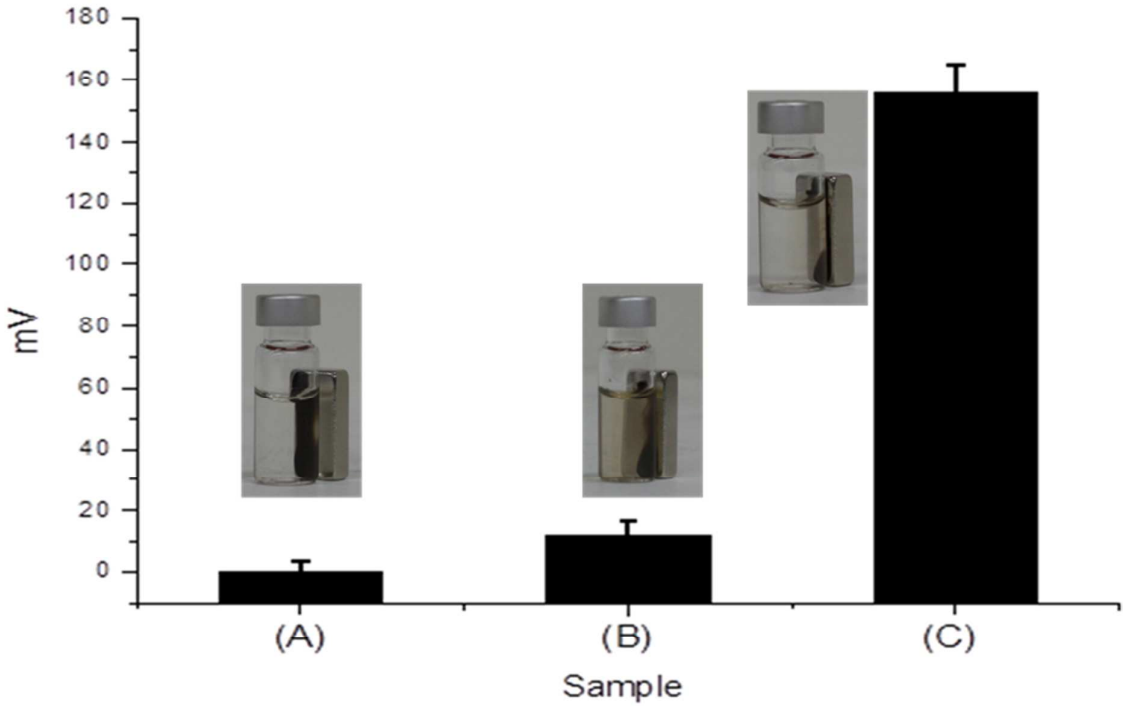


Figure 1. Measurement of (A) 500 nm diameter magnetic particles only, (B) mixture of 20 nm and 500 nm particles without Avidin (no biochemical linkage between the two magnetic particles), (C) mixture of 20 nm and 500 nm particles with Avidin. The measurement was performed after washing out the magnetic particles from the solution. For the calculation, the offset measured in (A) was subtracted from (B) and (C). The detailed experimental procedures are described in the text. All the experiments were triplicated.

FIGURE 2

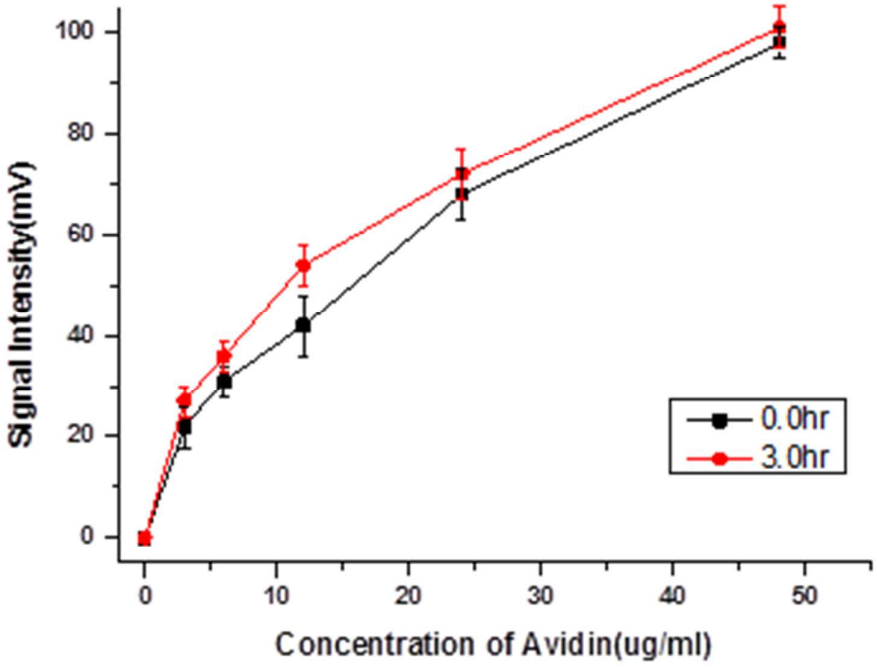
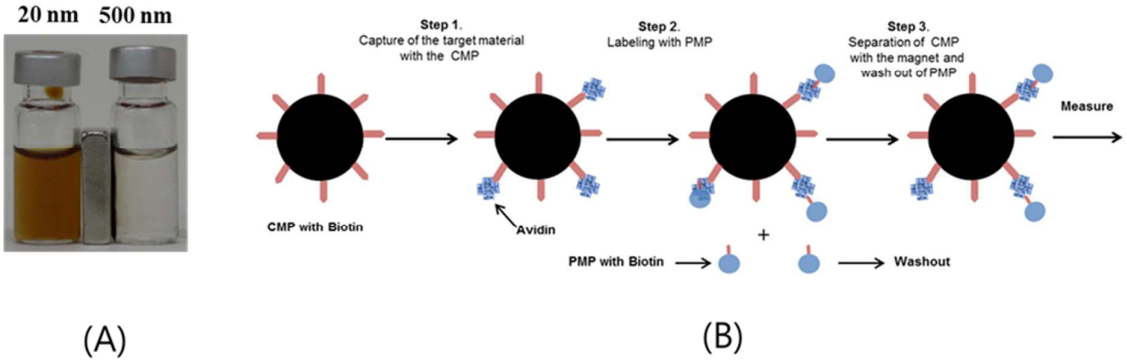


Fig. 2. Measurement of signal intensity as a function of Avidin concentration. The square dots denote the results from the measurement immediately after the reaction, the red circles were measured after 3 hours storage under usual atmosphere, showing the repeatability and stability of the method.

SCHEME 1



Scheme 1 (A) Photograph of the two different magnetic particle samples showing the different magnetization and basic principle of the experiment. (B) Schematic drawing of the analytical procedure. 20 nm diameter Probe Magnetic Particles (PMP) with a single magnetic domain are bound to Capturing Magnetic Particles (CMP, diameter 500 nm, with multiple magnetic domains) through a reaction between analyte and capturing agent immobilized on the surface of the CMP. In this experiment, the model compounds are Avidin and Biotin. The unbound PMP are washed out. Quantification is performed by the measurement of PMP bound to the CMP using the Frequency Mixing Magnetic Detection technique.