Electronic Supporting Information for

Gold-plated magnetic polymers for highly specific enrichment and label-free detection of blood biomarkers under physiological conditions

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Experimental section

Materials

Cytochrome C (C2037), sinapinic acid (85429), 1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC), *N*–hydroxysuccinimide (NHS), human serum (AB type), prostate specific antigen (PSA, P3235) and protein G recombinant (19459) were purchased from Sigma-Aldrich (St. Louis, MO). Carboxyl-terminated hexa(ethylene glycol) undecane thiol (CMT002) was obtained from Nanoscience Instruments (Phoenix, AZ). Myoglobin, creatine kinase-muscle brane (CK-MB, 30-AC65), rabbit polyclonal anti-myoglobin antibody (70-MR13), and myoglobin-free human serum (90R-110) were from Fitzgerald (North Acton, MA). Mouse monoclonal anti-PSA antibody (ab403, isotype: IgG1) and mouse monoclonal anti-CK-MB antibody (ab19603, isotype: IgG1) were obtained from Abcam (Cambridge, UK). Magnetic gold microspheres (MGMs) (M-NG0501, 15 μm in diameter, Nomadien.com) were constructed using proprietary methods including electroless plating of auric acids on proprietary magnetic poly(methylmethacrylate) (PMMA) beads (Nomadien.com). 15 μm carboxyl-functionalized polymer beads (PC07N) and 7.9 μm carboxyl-functionalized magnetic beads (UMC4N) were from Bangs Laboratories (Fishers, IN). 2.8 μm protein G-conjugated magnetic beads (Dynabeads, 10003D) were purchased from Life technologies (Carlsbad, CA). 2 μm protein G-conjugated magnetic beads (S1430S) were obtained from New England Biolabs (Ipswich, MA). PBS buffer solution (pH 7.4) was composed of 0.01 M phosphate buffered saline, 0.138 M NaCl, and 0.0027 M KCl. TTBS buffer solution (pH 7.4) was composed of 0.05% (v/v) Tween-20, 20 mM Tris-HCl and 150 mM NaCl.

Antibody conjugation of MGMs with orientation control

MGMs (0.63 mg) and absolute ethanol (0.5 mL) were added into a centrifuge tube and sonicated for a few seconds for dispersion. Then, MGMs were collected in the tube by a magnetic force while removing ethanol solution. MGMs were suspended in 0.5 mL of ethanolic solution containing 1 mM carboxyl-terminated alkanethiol to generate self-assembled monolayers (SAMs) for about 1 week at room temperature (RT) with gentle rotation. After the formation

of SAMs, MGMs were washed with distilled water several times by collecting MGMs with a magnetic force, pipetting off the unwanted solutions, and refilling distilled water into the tube respectively. EDC (2 mM) and NHS (5 mM) in 0.5 mL of MES (50 mM, pH 5.5) were added into MGMs to activate carboxyl groups for coupling with protein G. The solution was then incubated with gentle shaking for 30 min at RT and removed by magnetic separation. Protein G (10.5 μg in 85 μL MES) was allowed to bind covalently with the SAM-protected MGMs for 12 h at RT and washed with PBS (10 mM, pH 7.4) three times. Addition of IgG proteins (10.5 μg in 105 μL PBS) into the protein G-modified MGMs were followed and incubated for 2 h at RT. The antibody-conjugated MGMs were washed with PBS three times to achieve the complete removal of unbound excess antibodies. The resulting products were dispersed in a PBS solution and stored at 4 °C.

Antibody conjugation of MGMs without orientation control

SAM-protected MGMs (0.48 mg) was reacted with 2 mM EDC/5mM NHS in 0.5 mL MES buffer solution for 30 min at RT. The MGMs were magnetically collected while removing the supernatant. Then IgG proteins (4 μg in 40 μL of MES buffer solution) were immediately added into the centrifuge tube containing the MGMs for the conjugation of the IgG proteins to the MGMs via covalent bonds for overnight at RT. The antibody-conjugated MGMs were washed with PBS three times to achieve complete removal of unbound excess antibodies. The resulting products were dispersed in a PBS solution and stored at 4 °C.

Quantification of surface carboxy groups on microspheres

We followed a simple colorimetric method developed by Andreas Henning et al.[S1] Briefly, varying amounts of microsphere were incubated with 200 μM Ni^{2+} for 2 min in 10 mM HEPES buffer, pH 7.5 (total volume 0.6 mL). The solution was centrifuged at 16000 rcf for 1 min. 500 μL of the supernatant of the solution was diluted with 490 μL of HEPES buffer and then 10 μL of pyrocatechol violet (PV) was added (40 μM in the final volume 1 mL). The absorbance at 650 nm was plotted with respect to the amounts of microsphere stock solution. Linear fitting of the initial decrease gave the slope a and the y-intercept b. The number of surface carboxy groups obtained by the following equation

$$\text{surface carboxy groups } \left(\frac{\mu\text{mol}}{\text{particle}}\right) = \frac{n[\text{M}^{2+}]Va}{w(A_{\text{PV}} - b)}$$

where V is the volume and $[M^{2+}]$ the metal ion concentration, a the slope, b the y-intercept of the initial linear decrease, A_{PV} the absorbance of PV in the absence of M^{2+}, w the particle concentration (in particles mL^{-1}) of the stock solution, and n, 2.65, a stoichiometry factor indicating the number of surface carboxy groups per metal cation.

Immunoassays in human serum using antibody-modified MGMs

Antibody-conjugated MGMs (60 μg, ~49500 beads) were suspended in a 0.5 mL human serum containing a desired concentration of antigen and incubated for 30 min with gentle rotation at RT. Then, antigen-captured MGMs were washed with TTBS and water for 2 times respectively. Antigen-captured MGMs were mixed with 1.2 μL of matrix

solution (7.5–15 mg mL^{-1} sinapinic acid in 50:50:0.1 (v/v/v) acetonitrile, water, trifluoroacetic acid containing 5–10 fmol μL^{-1} cytochrome C as internal standard) and the mixture solution with all antigen-captured MGMs was pipetted onto the sample plate directly for MALDI-TOF MS analysis. Notably, myoglobin quantification was conducted using orientation-controlled antibody-conjugated MGMs via protein G affinity. Quantification of PSA and CK-MB were conducted using random-oriented antibody-conjugated MGMs.

Kinetic study of immunoreactions of antibody-conjugated MGMs and antigens

Antibody-conjugated MGMs (70 μg) were dispersed in 0.7 mL of sample solution (0.5 μg mL^{-1} Myo, 10 mM PBS, pH 7.4) with gentle rotation and allowed for immunoreactions at RT. At a desired time (3, 7, 12, 20, 30, and 60 min), 0.1 mL of sample solution containing the antigen-captured MGMs (10 μg) was transferred to a new centrifuge tube and immediately washed with PBS and water for two times each to stop further immunoreactions. Then, the antigen-captured MGMs were analyzed by MALDI-TOF MS.

Comparison of the MGMs modified with orientation-controlled antibodies with commercial beads

Carboxyl-functionalized magnetic (7.9 μm in diameter) and non-magnetic (15 μm in diameter) beads (Bangs Lab), protein G-modified magnetic beads (2 μm in diameter, New England BioLabs), and protein G-modified magnetic Dynabeads (2.8 μm in diameter, Life Technologies) were functionalized with Myo antibodies by following the exactly same procedures for the preparation of Myo antibody-conjugated MGMs (15 μg, ~12400 beads). All microspheres were reacted with 0.2 mL of human serum spiked with Myo (0.2 μg) for 30 min at RT. The reacted beads were washed with TTBS and water for two times, then analyzed by MALDI-TOF MS.

Comparison of the MGMs with random-oriented antibodies with commercial beads

Carboxyl-functionalized magnetic beads (7.9 μm in diameter, 178000 beads; 15 μm, 49500 beads, Bangs Lab) were functionalized with PSA antibodies by following the same procedures for the preparation of PSA antibody-conjugated MGMs (60 μg, ~49500 beads). All microspheres were immunoreacted with 0.5 mL human serum spiked with 5 ng PSA for 1 h at room temperature. The reacted beads were washed with TTBS and water for two times, then analyzed by MALDI-TOF MS.

Multiplex immunoassay

Each of Myoglobin, CK-MB, and PSA antibodies was conjugated to SAM-protected MGMs (15 μg) in a random orientation manner. Three types of antibody-conjugated MGMs (45 μg) were immunoreacted with a 0.5 mL human serum spiked with 25 ng Myo, 50 ng CK-MB, and 50 ng PSA for 30 min at RT. The antigen-captured MGMs were washed with TTBS and water for two times then applied to MALDI-TOF MS analysis.

MALDI-TOF mass spectrometry

Matrix solution (1.2 μL of 7.5–15 mg mL^{-1} sinapinic acid in 50:50:0.1 (v/v/v) acetonitrile, water, trifluoroacetic acid) was used to assist protein desorption and ionization from the bead surface. The mixture solution composed of the

matrix solution and target-captured beads was pipetted onto the sample plate directly for MALDI-TOF MS analysis. Mass analysis was carried out using a Voyager DE^{TM} Biospectrometry mass spectrometer (Applied Biosystems) with 337-nm nitrogen laser at the Seoul National University National Center for Inter-University Research Facilities (NCIRF). Typical experimental parameters were as follows: Linear mode of operation, positive polarity, 25000 VDC accelerating voltage, 92% grid voltage, 500-ns delayed extraction, and 2 kDa of low mass gate. For the quantifications of Myo, CK-MB and PSA in human sera, we used fixed laser intensity and repetition frequency (1455 and 20.0 Hz). Spectra were obtained by averaging ~400 shots followed by a Gaussian smoothing and baseline correction (optional) using Data-Explorer software (Applied Biosystems).

Field-Emission Scanning Electron Microscopy (FE-SEM)

The surface morphology of MGMs on a carbon tape was monitored using JSM 5410LV (JEOL, Japan) with 2 kV of acceleration voltage at the National Instrumentation Center for Environmental Management (NICEM) of Seoul National University.

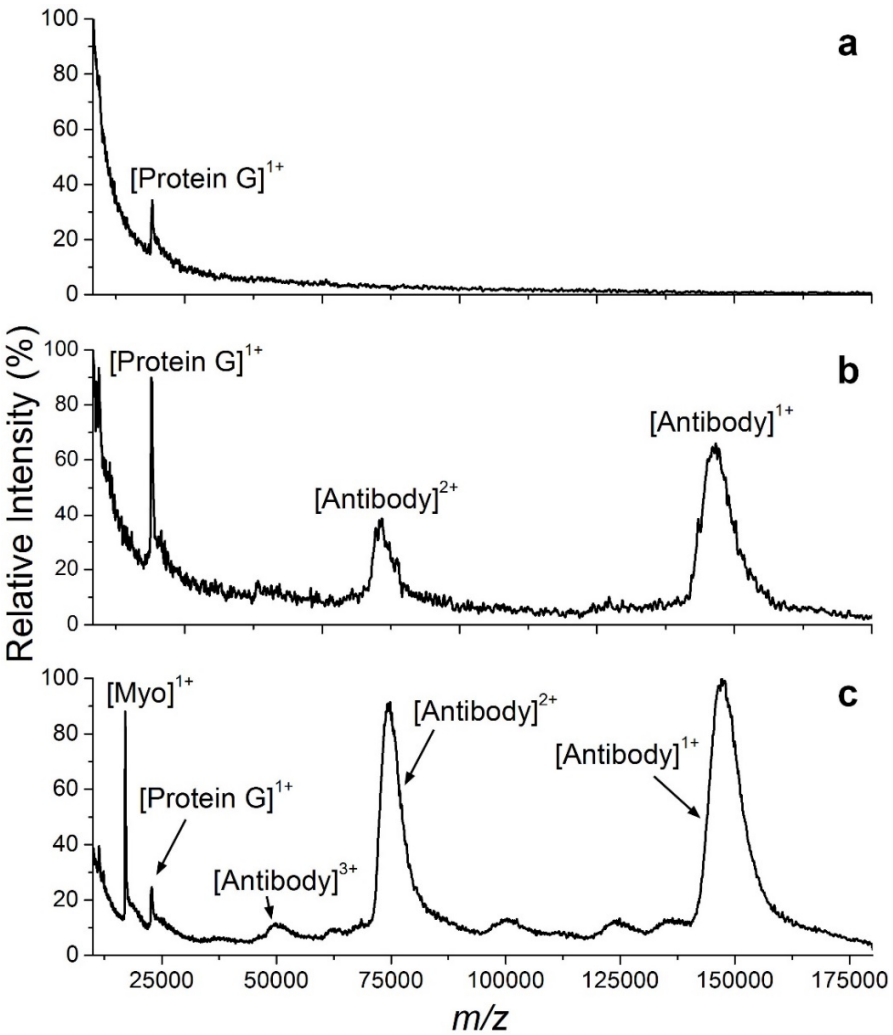


Fig. S1 MALDI-TOF mass spectra of MGMs modified with protein G (a), antibody (b), and Myo enriched from a PBS solution (c).

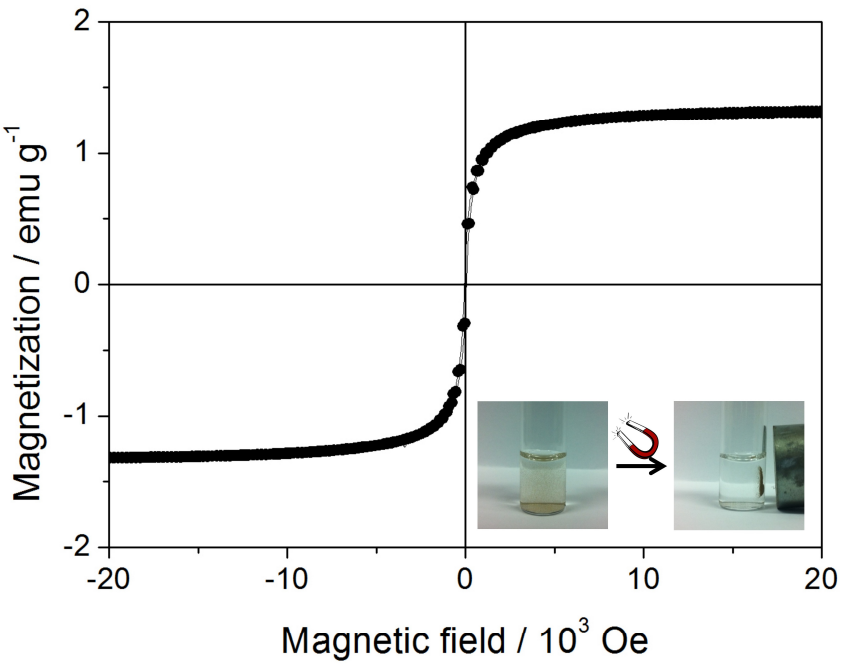


Fig. S2 The hysteresis loop of gold-plated magnetic microspheres after antibody immobilization.

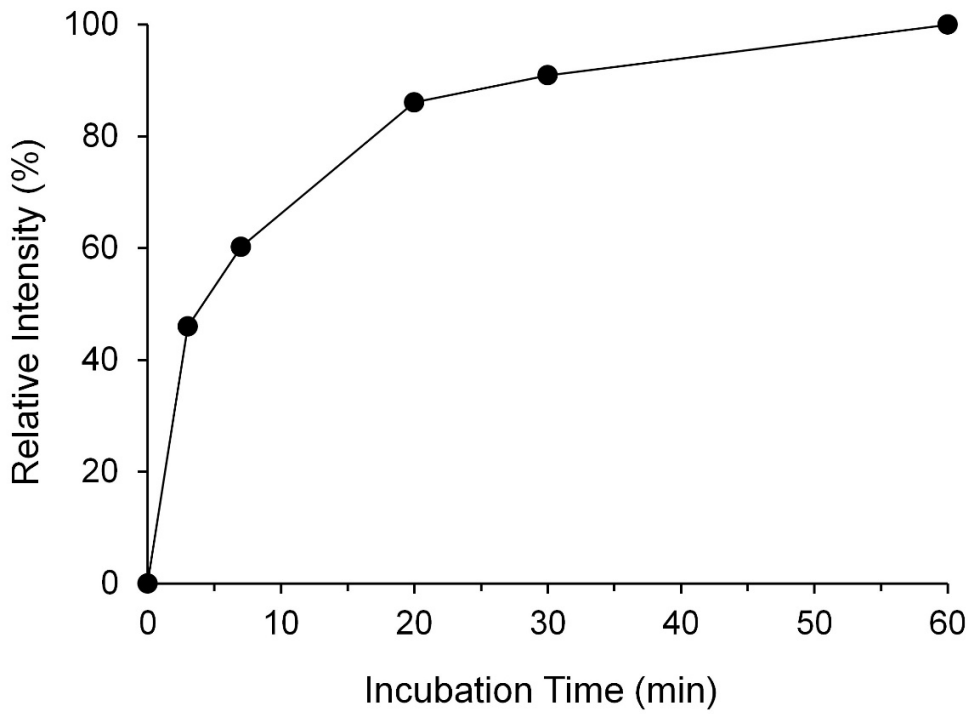


Fig. S3 Effect of incubation time on antibody-antigen immunoreaction using antibody-conjugated MGMs. After incubation (3, 7, 12, 20, 30, and 60 min) of Myo antibody-conjugated MGMs in the PBS solution composed of 500 ng/mL Myo, the quantities of the Myo extracted by antibody-conjugated MGMs were detected by MALDI-TOF MS. The relative peak intensity generated using the ratio of the ion intensity of Myo to the ion intensity of CytC was plotted as a function of incubation time.

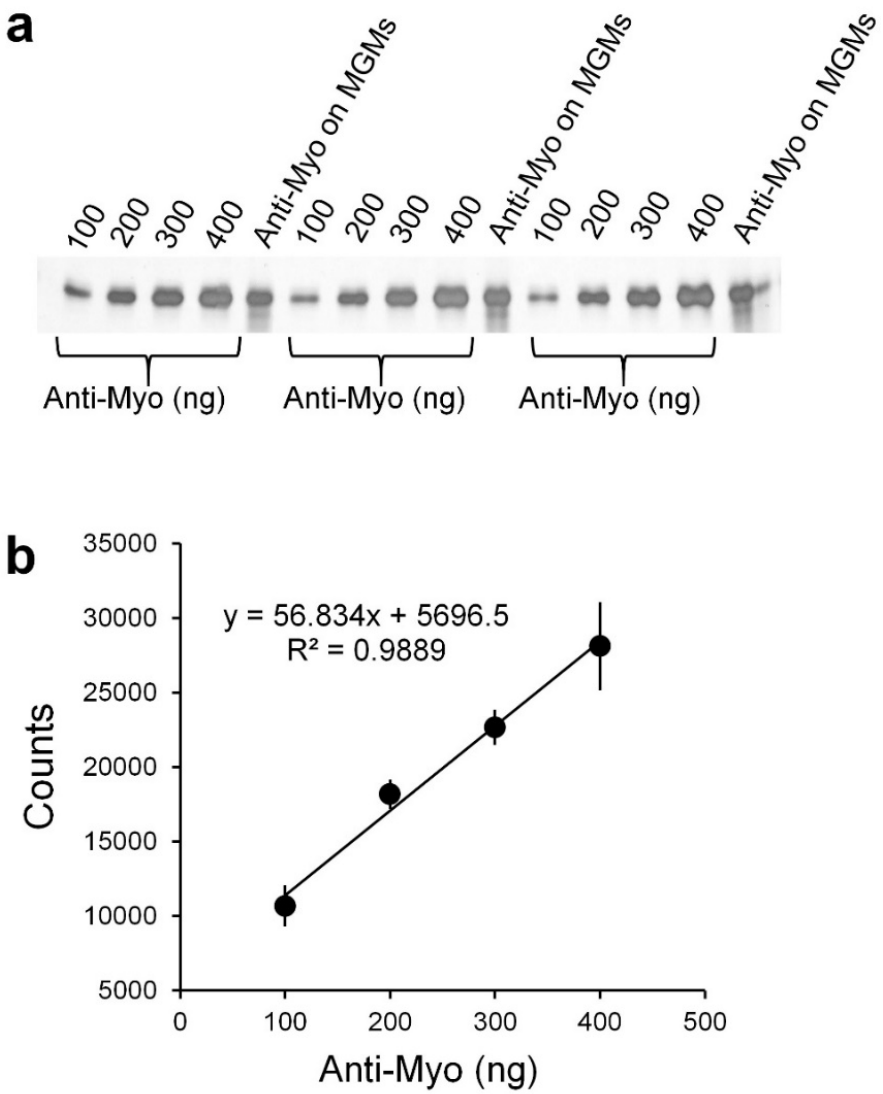
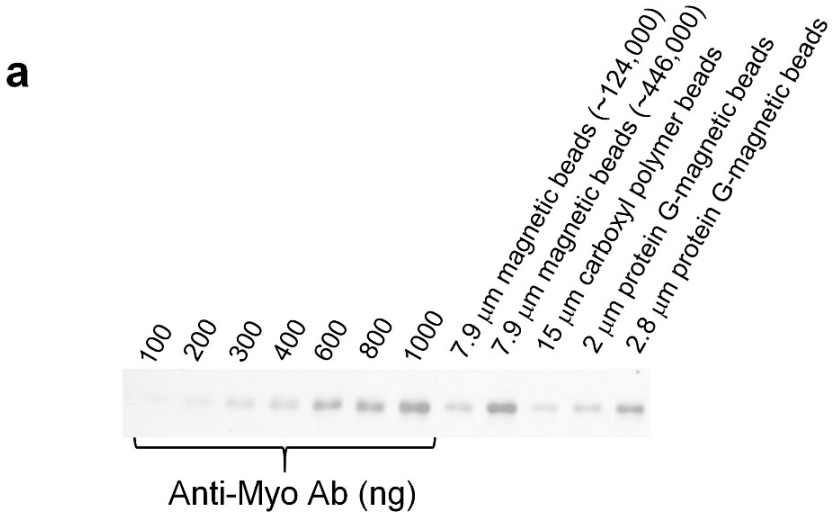
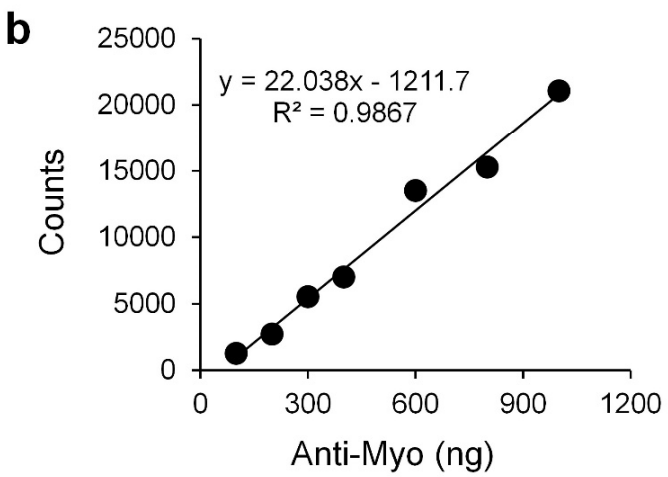


Fig. S4 Quantification of antibodies immobilized on MGMs. a) A silver-stained gel image after SDS-PAGE showing 50 kDa heavy chains of IgG. b) Calibration plot calculated from the density of protein bands and the error bars represent the standard deviations of three independent measurements.

In theory, the surface area of the ~12400 MGMs is 8.77×10^{-6} m^2. If all the antibodies (spheroid shape with the dimensions of 15 nm × 15 nm × 3 nm) are packed in upright position, the maximum amount per ~12400 MGMs would be 57 ng (See *Principles in Adsorption to Polystyrene*, Peter Esser, M.Sc., Thermo Fisher Scientific Bulletin No. 6a. 2010).





c

Bead type (number used)	Antibody (ng)
15 μm MGMs (~12,400)	31
7.9 μm carboxyl-terminal magnetic (~12,400)	33
7.9 μm carboxyl-terminal magnetic beads (~44,600)	95
15 μm carboxyl-terminal polymer beads (~12,400)	22
2 μm protein G magnetic beads (NEB)	33
2.8 μm protein G magnetic beads (Dynabeads)	68

Fig. S5 Quantification of antibodies immobilized on the testing beads. a) A silver-stained gel image after SDS-PAGE showing 50 kDa heavy chains of IgG. b) The calibration plot obtained from the pixel density of the IgG bands using Image J software. c) The amount of antibodies immobilized on each microbeads is tabulated.

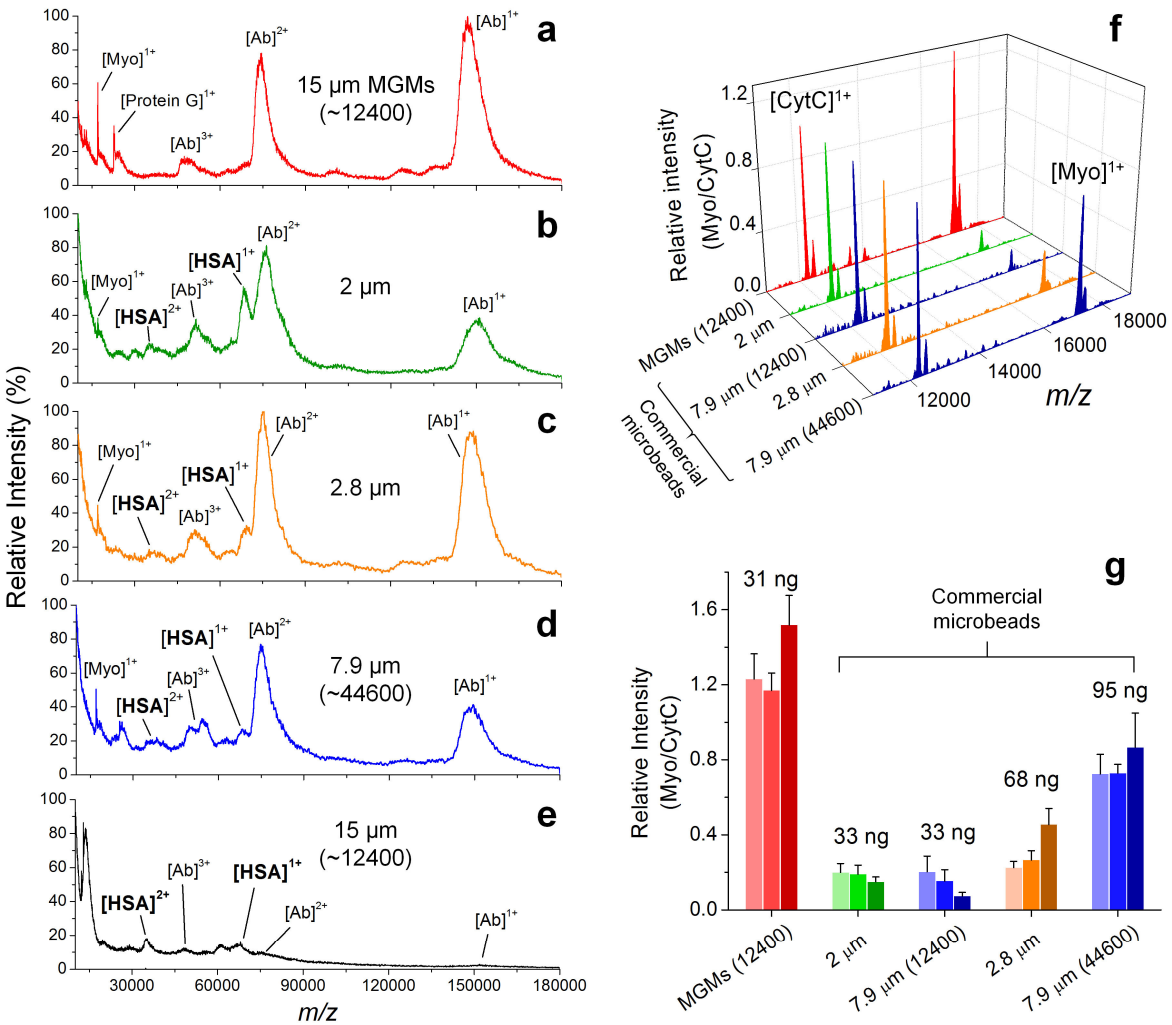


Fig. S6 a-e) Evaluation of nonspecific adsorption and detection of Myo using MGMs in comparison with other microbeads. Representative MALDI-TOF mass spectra of microbeads after incubation with 0.2 mL of human serum spiked with 0.2 μg of Myo. a) MGMs (~12400 beads, 15 μm), b) protein G magnetic beads (2 μm; NEB), c) protein G magnetic beads (2.8 μm; Dynabeads), d) magnetic carboxyl beads (~44600 beads, 7.9 μm; Bangs Laboratories), and e) carboxyl beads (~12400 beads, 15 μm; Bangs Laboratories). f) Representative MALDI-TOF mass spectra for the affinity extraction and detection of Myo using MGMs and other microbeads. Cytochrome C (CytC) served as an internal standard. g) Comparison of three sets of independent experiments. The amount of antibody immobilized on microbeads was annotated in nanograms. The error bars represent the standard deviations obtained from four to six spectra. Ab, antibody; HSA, human serum albumin.

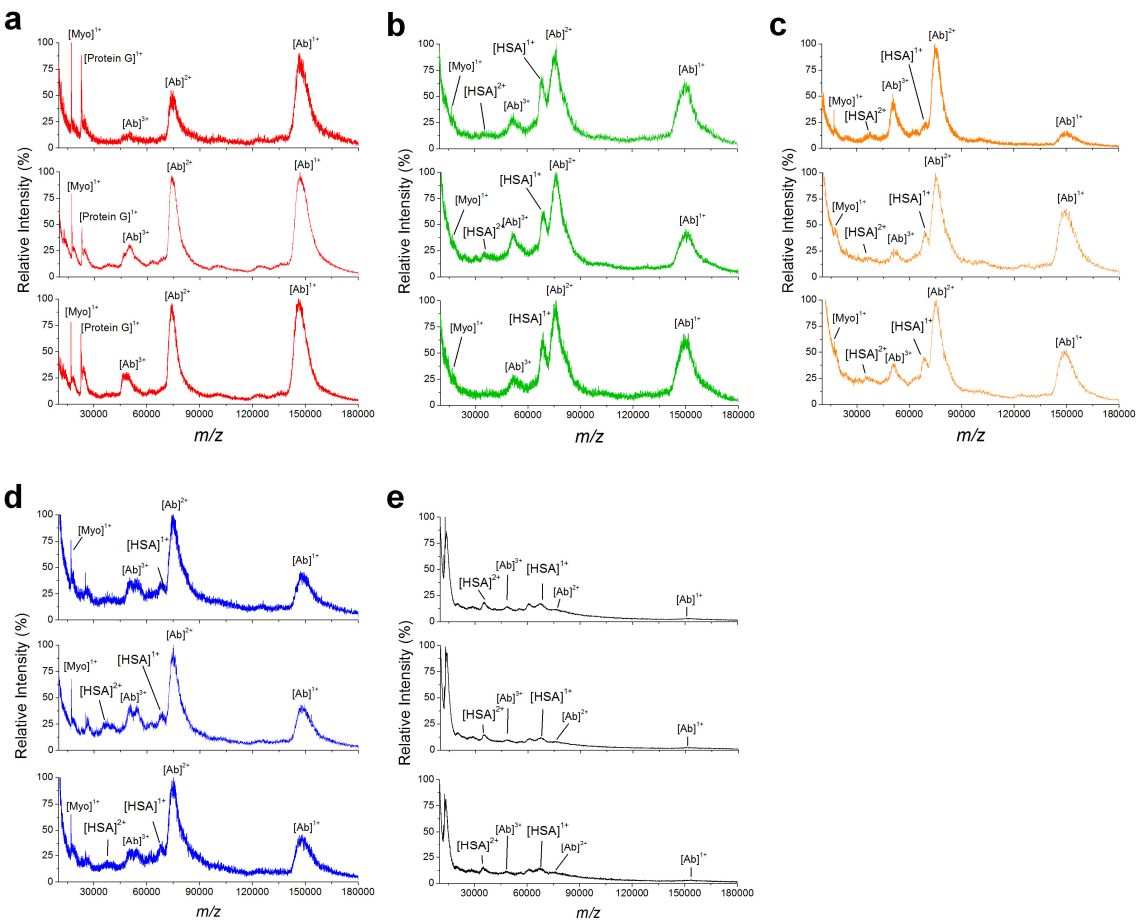
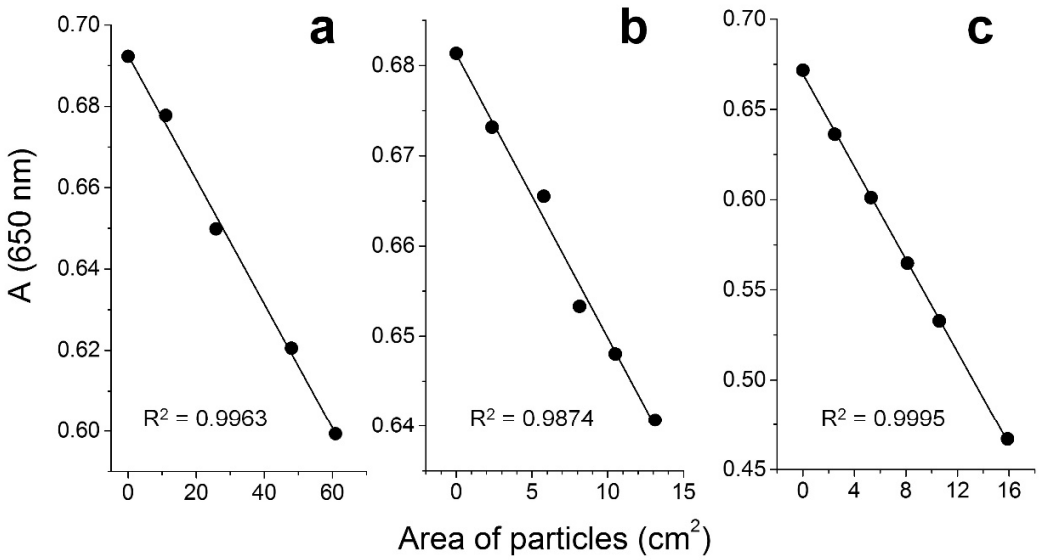


Fig. S7 Additional representative MALDI-TOF mass spectra of microbeads after incubation with 0.2 mL of human serum spiked with 0.2 μg of Myo. a) MGMs (~12400 beads, 15 μm), b) protein G magnetic beads (2 μm), c) protein G magnetic beads (2.8 μm), d) magnetic carboxyl beads (~44600 beads, 7.9 μm), and e) carboxyl beads (~12400 beads, 15 μm) with many unknown peaks. Spectra were obtained through two days of independent experiments. Ab, antibody; HSA, human serum albumin.



d

Bead type	Carboxyl group (molecules/cm^2)
15 μm polymer beads	4.3×10^{14} (5.1×10^{15} in the certificate of analysis)
15 μm MGMs	9.2×10^{14}
7.9 μm magnetic beads	3.8×10^{15}

Fig. S8 Determination of the amounts of surface carboxyl group. Plot of the absorbance at 650 nm versus the area of 15 μm polymer beads (a), 15 μm MGMs (b), and 7.9 μm magnetic beads (c). d) The density of surface carboxyl groups of each microbeads is tabulated.

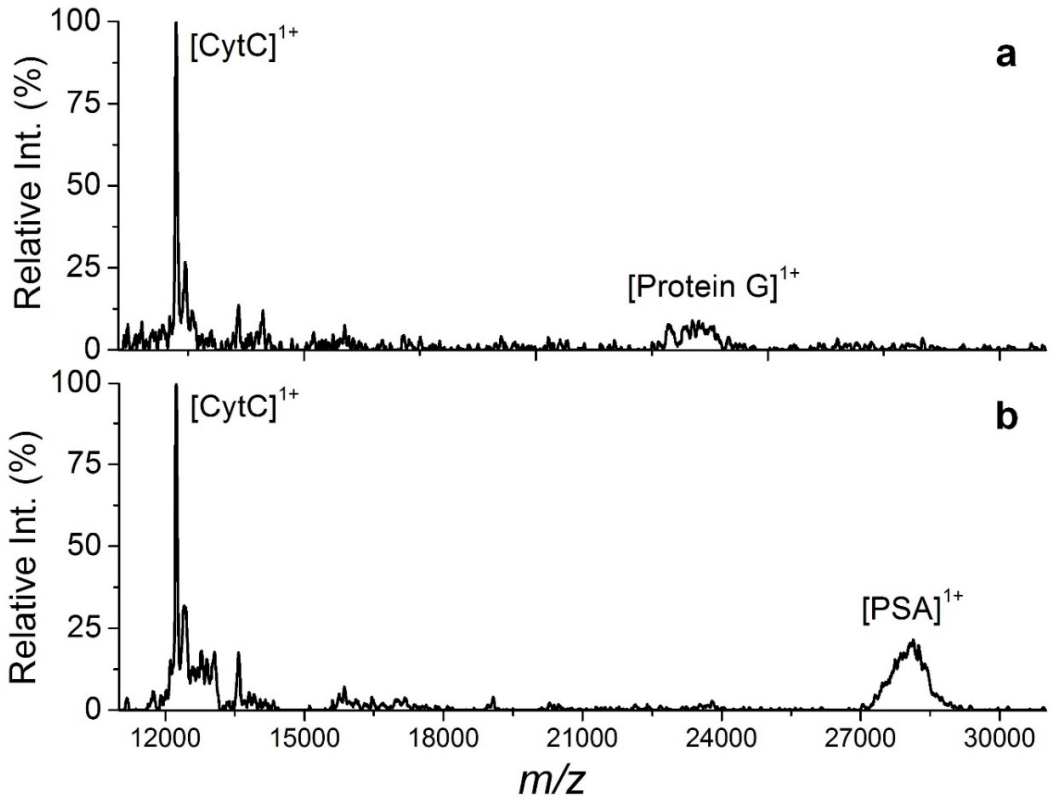


Fig. S9 Representative baseline-corrected MALDI-TOF mass spectra of antibody-modified MGMs after immunoreactions with 0.5 mL human serum spiked with 50 ng PSA. a) The IgG proteins were immobilized onto MGMs in an orientation-controlled manner via protein G affinity. b) The IgG proteins were conjugated to the SAMs on MGMs in a random orientation manner via covalent bond.

Unlike rabbit IgG that has strong binding to protein G, the IgG1 isotype of mouse anti-PSA antibody has moderate affinity to protein G.
(http://www.millipore.com/techpublications/tech2/binding_properties, accessed Feb. 2014)

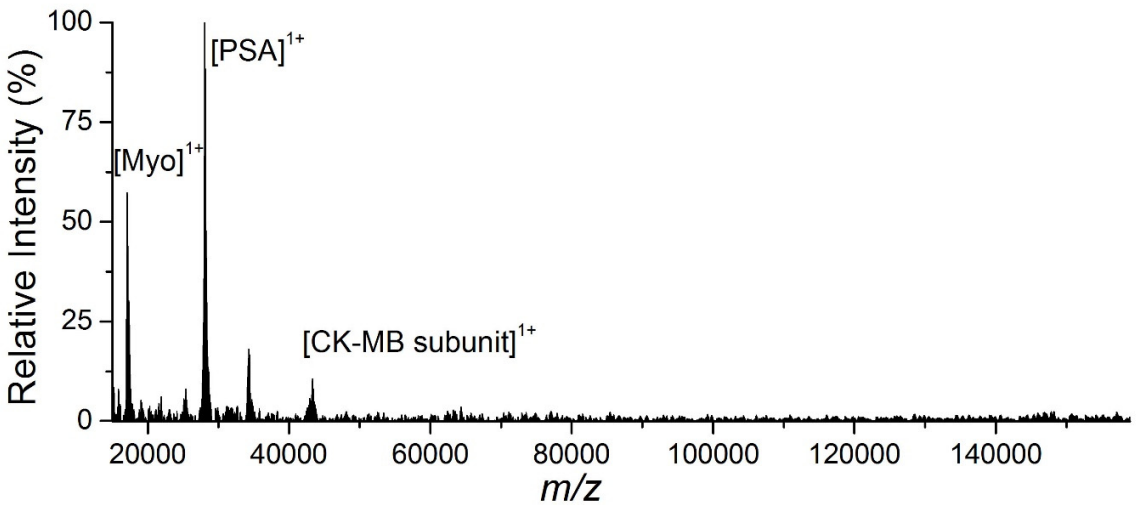


Fig. S10 Baseline-corrected whole-range MALDI-TOF mass spectra for multiplexed detection of Myo, CK-MB, and PSA.

Table S1. Detection limits and signal to noise ratios of the three biomarkers quantified using the MGMs and MALDI-TOF MS.

	Biomarkers (ng/mL)		
	Myo	CK-MB	PSA
Upper limit of quantification	200	250	100
Limit of detection[†]	0.5	10	25
Signal to noise[‡]	13	8.2	19

[†]Empirical values well above the blank signal + 3SD (standard deviation)

[‡]Based on root-mean-square (RMS)

[S1] A. Hennig, A. Hoffmann, H. Borcherding, T. Thiele, U. Schedler and U. Resch-Genger, *Anal. Chem.*, 2011, **83**, 4970-4974.