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ORIGINAL RESEARCH

Facile one-step coating approach to magnetic submicron particles with poly(ethylene glycol) coats and abundant accessible carboxyl groups

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Purpose: Magnetic submicron particles (MSPs) are pivotal biomaterials for magnetic separations in bioanalyses, but their preparation remains a technical challenge. In this report, a facile one-step coating approach to MSPs suitable for magnetic separations was investigated.

Methods: Poly(ethylene glycol) (PEG) was derived into PEG-bis-(maleic monoester) and maleic monoester-PEG-succinic monoester as the monomers. Magnetofluids were prepared via chemical co-precipitation and dispersion with the monomers. MSPs were prepared via one-step coating of magnetofluids in a water-in-oil microemulsion system of aerosol-OT and heptane by radical co-polymerization of such monomers.

Results: The resulting MSPs contained abundant carboxyl groups, exhibited negligible nonspecific adsorption of common substances and excellent suspension stability, appeared as irregular particles by electronic microscopy, and had submicron sizes of broad distribution by laser scattering. Saturation magnetizations and average particle sizes were affected mainly by the quantities of monomers used for coating magnetofluids, and steric hindrance around carboxyl groups was alleviated by the use of longer monomers of one polymerizable bond for coating. After optimizations, MSPs bearing saturation magnetizations over 46 emu/g, average sizes of 0.32 μm, and titrated carboxyl groups of about 0.21 mmol/g were obtained. After the activation of carboxyl groups on MSPs into N-hydroxysuccinimide ester, biotin was immobilized on MSPs and the resulting biotin-functionalized MSPs isolated the conjugate of streptavidin and alkaline phosphatase at about 2.1 mg/g MSPs; streptavidin was immobilized at about 10 mg/g MSPs and retained 81% ± 18% (n = 5) of the specific activity of the free form.

Conclusion: The facile approach effectively prepares MSPs for magnetic separations.

Keywords: magnetic submicron particles, carboxyl groups, PEG-bis-(maleic monoester), monomer, radical co-polymerization, steric hindrance

Introduction

Magnetic particles after functionalization with specific biomolecules are indispensable and precious biomaterials for magnetic separations of interacting biomolecules in mixtures, and are widely used for chemiluminescence immunoassay, extraction of nucleic acid, high-throughput screening of mixture-based ligand libraries, pulling-down analysis of interacting molecules, and so on.[1–10] For such applications in vitro, magnetic particles are preferable to display submicron to micron sizes and are thus denoted magnetic submicron particles (MSPs). In general, MSPs are required to concomitantly have abundant reactive groups for biomolecule immobilization, hydrophilic surfaces bearing negligible nonspecific adsorption of common substances, high saturation magnetization for efficient separation, good suspension stability,

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and stable chemical structures. Moreover, biomolecules immobilized on MSPs should display their maximal possible activities. Steric hindrance is the primary negative factor of activities of immobilized biomolecules, and long flexible arms are required to anchor reactive groups for biomolecule immobilization. Additionally, MSPs for screening mixture-based ligand libraries are further required to have negligible extractable small compounds.[2–7,9–10] Consequently, it is preferable to develop facile approaches to MSPs that concomitantly have as many of those favorable properties as possible.

In classical approaches to MSPs, synthetic magnetofluids are coated via radical polymerization of hydrophobic monomers in oil-in-water microemulsion systems.[11–23] However, those classical approaches tolerate the labor and face the technical challenges to engineer both hydrophilic surfaces for negligible nonspecific adsorption of common substances and flexible long arms bearing reactive groups for biomolecule immobilization.[19–23] In theory, the properties of monomers primarily determine the tendency of MSP surfaces or coats to exert nonspecific interactions with common substances. Thus, one-step coating of magnetofluids via radical polymerization of hydrophilic linear monomer(s) in a water-in-oil microemulsion system may be an ideal solution to the challenges associated with those classical approaches. In this one-step approach, a preferable monomer is a hydrophilic linear polymer bearing a carbon–carbon double bond at one end for polymerization and another type of reactive group at the other end for biomolecule immobilization. After polymerization, the polymer chain directly serves as the flexible arm to anchor the reactive group for biomolecule immobilization. A long hydrophilic linear monomer bearing two such types of reactive groups at both ends still can be utilized, since the large motion freedom of monomer ends will provide some unpolymerized ones to immobilize biomolecules so that the polymer chains still serve as the flexible arms. On the other hand, the abundance of reactive groups of negligible steric hindrance on the surfaces of MSPs for biomolecule immobilization should be as large as possible for reasonable binding capacities of MSPs after functionalization with biomolecules. Hence, there should be some additional forces to drive reactive groups on the ends of the hydrophilic linear monomers to the MSP surfaces during the coating of magnetofluids.

Poly(ethylene glycol) (PEG) is a hydrophilic linear polymer that has one hydroxyl group at each end and negligible nonspecific interactions with common substances.[20,23–26] Unsaturated derivatives of PEG chains bearing carboxylic acids at two ends may serve as monomers for this one-step approach (Figure 1). Among carboxyl groups from PEG derivatives as monomers in aqueous phases, there are electrostatic repulsions during polymerization to facilitate driving carboxyl groups to the surfaces of MSPs. To drive more carboxyl groups to the surfaces of MSPs, such electrostatic repulsions can be enhanced by increasing the concentrations of carboxyl groups from monomers

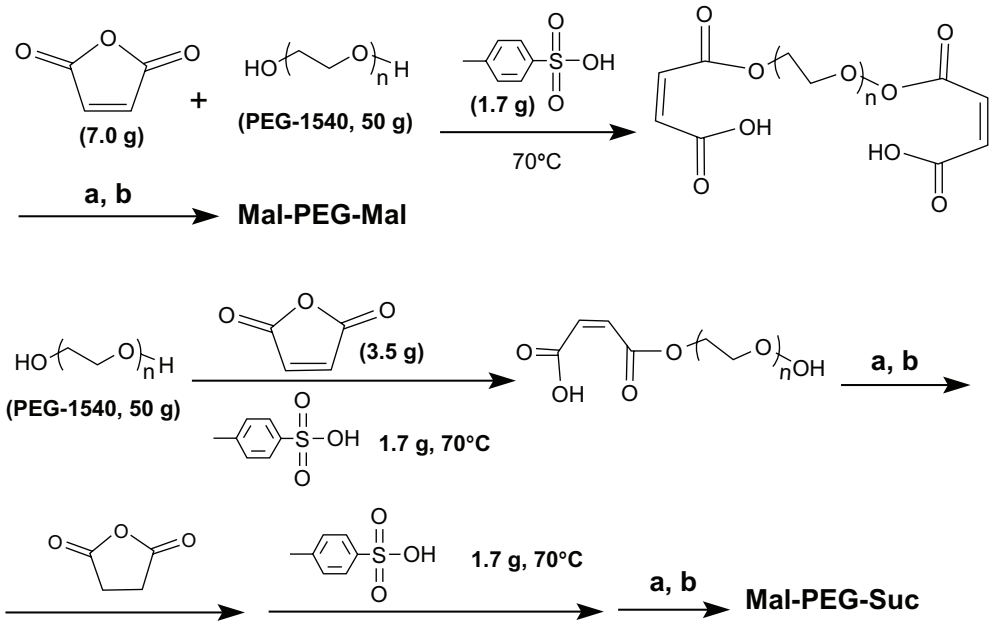

Figure 1 Preparation of PEG derivatives as monomers. (**a**) Precipitation and wash with ethyl ether. (**b**) Dissolution in THF and precipitation in ethyl ether, repeatedly.
Abbreviations: PEG, poly(ethylene glycol); THF, tetrahydrofuran.

and/or pH values of the aqueous phases. For compact coats and favorable polymerization rates, the increase in the concentrations of carboxyl groups from monomers is preferable. Solution viscosity of PEG derivatives limits their maximal concentrations in aqueous phases and the use of unsaturated PEG derivatives bearing two carboxyl groups at both ends as monomers is more practical. On the other hand, for lowering steric hindrance around carboxyl groups, longer PEG derivatives bearing carboxyl groups at both ends but just one carbon–carbon double bond for polymerization can be utilized as monomers. Indeed, the one-step approach with such special monomers yielded MSPs concurrently bearing many properties favorable for applications to magnetic separations. Here, we report this one-step approach to such MSPs.

Materials and methods

Instruments, apparatus, and chemicals

Instruments and apparatus were used with standard protocols, including a Shimadzu XRD6000 X-ray diffractometer (Shimadzu Corporation, Kyoto, Japan), a Nicolet Magna 550 series II Fourier transform infrared spectroscopy (FTIR) spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), an SDT-Q600 Thermogravimetric analyzer (TA Instruments, New Castle, DE, USA), an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA), a Biotek ELX 800 microplate reader (BioTek, Winooski, VT, USA), a Hitachi S-3000 N scanning electronic microscope (Hitachi Ltd, Tokyo, Japan), a Hitachi-7500 transmission electronic microscope, a Zetasizer Nano ZS90 Size Distributions and Zeta potential Meter (Malvern Instruments, Malvern, UK), and an LDJ 9600-1 vibrating sample magnetometer (LDJ Electronics Inc, Troy, MI, USA). Samples for X-ray diffraction, FTIR, saturation magnetization, and thermogravimetric analysis (TGA) analyses were all prepared by drying MSPs at 70°C for 12 hours, followed by grinding. TGA was performed at 10°C/minute in air or nitrogen atmosphere. A Promega PolyATract® System 1000 Stand (Promega Corporation, Madison, WI, USA) and Mapada UV1600 spectrophotometer (Mapada Instruments, Shanghhai, People's Republic of China) were used routinely.

All chemicals were from Aladdin Reagents (Shanghai) Inc, (Shanghai, People's Republic of China) unless otherwise stated.

Preparation of PEG derivatives as monomers

PEG was reacted with maleic anhydride in threefold excess at 70°C in the absence of any solvent in a water bath (all the used chemicals were melted at this temperature) to produce PEG-bis-maleic monoester (Mal-PEG-Mal-x, x when used indicates the average molecular weight of PEG).[26,27] The resulting Mal-PEG-Mal after cooling was dissolved in tetrahydrofurane (THF) and precipitated in ethyl ether; this process was repeated three times to purify the Mal-PEG-Mal (Figure 1). The reaction of PEG with maleic anhydride at a 1:1 molar ratio at 70°C in the absence of any solvent produced PEG-mono-(maleic monoester), which, without purification, was further reacted with excessive succinic anhydride in melted states to produce mixed monoesters of PEG (Mal-PEG-Suc-x). Mal-PEG-Suc-x was purified via the procedure for purifying Mal-PEG-Mal. After drying in a vacuum at 60°C for 12 hours, molar quantities of the monomers were calculated with their masses and their average molecular weights.

Preparation of magnetofluids

$FeCl_2 \cdot 4H_2O$ at 1.0 g and $FeCl_3 \cdot 6H_2O$ at 1.5 g were dissolved in 100 mL of water degassed by nitrogen bubbling in a three-necked flask. Under vigorous mechanical stirring and continuous nitrogen flow at 25°C, 10 mL of concentrated aqueous ammonia was added.[20] Immediately, an aqueous solution in 5 mL containing 2.0 g of a monomer was added dropwise to disperse magnetic cores; the resulting mixture was kept under continuous vigorous mechanical stirring and nitrogen flow for 20 minutes. Then, the mixture was rapidly heated to 70°C and was kept at 70°C for 30 minutes. Finally, the mixture was cooled by tap water; magnetofluids were separated by a Promega PolyATract® System 1000 Stand (this magnetic separator was used throughout this study), and further washed three times with water. Magnetofluids were quantified by volumes of precipitates under external magnetic fields or by mass weights after drying in a vacuum at 70°C for 12 hours. Magnetofluids dispersed with Mal-PEG-Mal-400 were denoted magnetofluids400; magnetofluids dispersed with Mal-PEG-Mal-1540 were designated magnetofluids1540.

Coating of magnetofluids in microemulsion system

Under mild mechanical stirring, magnetofluids of about 0.7 mL were dispersed in 3.0 mL of water, and then mixed with 2.0 g or 4.0 g of a monomer or a mixture of two monomers dissolved in 5.0 mL of water plus a 1.0 mL solution of N,N′-methylene bis-acrylamide saturated at 25°C. About 10 mL of this mixture was dispersed in 500 mL of heptane solution containing 12 g of aerosol-OT (AOT; Sigma-Aldrich, St Louis, MO, USA) under 2000 rpm stirring for 20 minutes to

make a microemulsion. Then, a 1.0 mL solution of ammonium peroxydisulfate saturated at 25°C and a 1.0 mL aqueous solution of 0.5% N,N,N′,N′-tetramethylethylenediamine were added consecutively to initiate radical co-polymerizations at 37°C. The polymerization reaction lasted for 6 hours, unless otherwise stated, under 2000 rpm stirring and continuous nitrogen flow. The resulting MSP, referred to as MSP-PEG-COOH hereafter, was magnetically separated, washed with a mixture of acetone and methanol at 9:1 and then with THF, each for three times, and finally suspended in 10 mM sodium phosphate buffer at pH 7.4 or water at 10% (v/v) for storage. The quantities of MSP-PEG-COOH were indexed by volumes of packed precipitates in a magnetic field or by mass weights after drying at 70°C for 12 hours. Viscosity of longer monomers limited their final quantities to 0.5 g per mL in water to make the microemulsion in 500 mL of heptane of 12 g AOT. Hence, the effects of just two quantities of monomers on the properties of MSP-PEG-COOH were tested.

Suspension stability and nonspecific adsorption

MSPs or magnetofluids of about 25 mg were suspended in the phosphate buffer at pH 7.4 to have absorbance of about 1.0 ± 0.05 at 570 nm. Then, the suspension in 2.2 mL was transferred into a glass cuvette of 4.0 mL; suspension transmittance was recorded at 570 nm at 25°C.

Methyl 4-nitrobenzoate ($C_8H_7O_4N$) was from Alfa Aesar (Ward Hill, MA, USA); 4-nitro-1-naphthyl benzoate ($C_{17}H_{13}O_4N$) and 4-nitro-naphthyl-1-(n)-octyl ester ($C_{18}H_{23}O_4N$), were synthesized with corresponding acylchlorides and phenols, respectively. These three aromatic esters plus tryptophan with their logPs from −1.8 to about 5.7 calculated with ACDfree 11.0 (Advanced Chemistry Development, Inc, Lansing, MI, USA) were used as representative compounds. Packed precipitates of MSP-PEG-COOH of about 50 mg were suspended in 8.0 mL of sodium phosphate buffer at pH 7.4; an aliquot of 1.0 mL of the suspension was withdrawn and mixed with an indicated compound in 20 μL of THF. After mixing for 30 minutes under continuous mild shaking, MSPs were separated by magnetic forces and the supernatant was removed as completely as possible (usually residual supernatant was about 0.12 mL). Bound compounds were extracted with 60 μL of THF at 45°C. Such solutions of bound compounds were then analyzed with a Merck LiChrospher 100 C_{18} reverse column (10.0 cm × 0.46 cm; Merck and Co, Inc, Whitehouse Station, NJ, USA) on an Agilent 1100 liquid chromatography system using an Agilent 1365B absorbance detector.[10] The mobile phase was methanol-water (8:2). Elution was monitored by absorbance at 254 nm.

Structural stability of MSP

MSP-PEG-COOH or magnetofluids of about 25 mg were suspended in 2.5 mL of an indicated buffer at 0.20 M and mixed continuously. At an indicated time, magnetic fields were used to produce the supernatant, and 50 μL of supernatant was withdrawn to quantify ferrous ion with 1,10-phenanthroline after reduction by hydroxylamine. In detail, such a sample of 50 μL was mixed with a 50 μL solution of 10% hydroxylamine hydrochloride and kept at 25°C for 30 minutes; a 250 μL solution of 10% sodium acetate and a 100 μL solution of 0.15% 1,10-phenanthroline were added for reaction of 15 minutes at 25°C before the assay of absorbance at 510 nm.

Titration and activation of carboxyl groups

MSP-PEG-COOH was washed five times with water and then dried in a vacuum at 70°C for 12 hours. Quantities of carboxyl groups were estimated via titration against a 5.0 mM NaOH solution; the equivalency point of titration was that for pH 7.8 (the pH for 5.0 mM of sodium acetate) with a PHS-3C pH meter (INESA Scientific Instrument Co, Ltd, Shanghai, People's Republic of China). MSP-PEG-COOH was washed repetitively with THF at 45°C. Carboxyl groups were activated by reaction with N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide in THF at 45°C for 6 hours.[9,10,28,29] The resulting MSPs were denoted as MSP-PEG-CO-NHS hereafter and washed five times with THF before the conjugation reaction.

Binding capacity of immobilized biotin

Mono-biotinyl-ethylenediamine was prepared with NHS-activated biotinyl ester and ethylenediamine in great excess in anhydrous dichloromethane[9,10,28,29] and was reacted in great excess in anhydrous dimethylformamide with MSP-PEG-CO-NHS. The resulting biotinylated MSPs were washed repeatedly with 10 mM of Tris-HCl buffer at pH 7.4. Then, the conjugate of streptavidin (SAV) and calf intestinal alkaline phosphatase (SAV-CIAP; Promega) in lysate of *Escherichia coli* BL21 (DE3) in 10 mM of Tris-HCl buffer at pH 7.4 was isolated with biotinylated MSPs. Bound SAV-CIAP was quantified in 0.50 mL of diethanolamine buffer (1.0 M at pH 9.8) with 10 mM of p-nitrophenylphosphate (Sigma-Aldrich); product absorbance was measured at 405 nm on a Biotek ELX 800 microplate reader with 0.15 mL of supernatant in a well.

The response plot of absorbance change rates to quantities of SAV-CIAP was checked with SAV-CIAP every day.

Binding capacity of immobilized SAV

SAV (Promega) was immobilized on MSP-PEG-CO-NHS at 25°C in 10 mM of sodium phosphate buffer at pH 8.0. In detail, a total of about 50 mg of packed precipitates of such activated MSPs after a rapid wash with cold water was suspended in 2.0 mL of cold 10 mM sodium phosphate buffer at pH 8.0. To an aliquot of 0.40 mL of the suspension, an indicated quantity of SAV in 0.60 mL of the same phosphate buffer was added; the mixture was kept at 25°C and shaken at 5 minute intervals for 50 minutes. The conjugates of the SAV and MSPs were separated by magnetic force and then suspended in 2.0 mL of 10 mM sodium phosphate buffer at pH 7.4 to estimate their binding capacity. Residual SAV in the supernatant was quantified as follows. The difference in SAV added and that leftover was that immobilized on the MSPs.

To quantify the SAV, the probe, N-(biotinyl)-N′-(1-naphthyl)-ethylenediamine (BNEDA, $C_{22}H_{28}N_4O_2S$), was prepared as described previously.[9,10,28,29] Residual SAV in the supernatant was quantified with BNEDA based on Förster resonance energy transfer using tryptophan residues as intrinsic donors and the bound BNEDA as the acceptor.[28,29] The excitation was made at 280 nm to measure fluorescence of the bound BNEDA at 430 nm due to the energy transfer.

The binding capacity of the SAV immobilized on the MSPs for BNEDA was estimated by the difference in BNEDA added and residual BNEDA after magnetic separation of the bound parts. The SAV immobilized on MSP-PEG-COOH at an indicated volume of suspension was incubated with 0.24 mM BNEDA, and then residual BNEDA in supernatant was quantified by its own fluorescence at 442 nm via excitation at 325 nm after magnetic separation of the bound BNEDA. Background signals from contaminants in samples were measured in the absence of BNEDA and were corrected. Data were determined in triplicate with coefficients of variations below 20%.

Results and discussion

Preparation of MSPs

Monomer structures were supported by C^{13} NMR. data, Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometer (MALDIFI-TOF-MS), and titration of COOH groups against 5.0 mM of NaOH solution (Figure S1). MSP-PEG-COOH was facilely prepared via the one-step approach. Solution viscosity of PEG derivatives limits the concentrations of long monomers to below 6.0 g in aqueous solutions for making microemulsion. In general, the yield of MSP-PEG-COOH each time was about 0.80 mL of packed precipitate (about 0.20 g after drying at 70°C for 12 hours) from 500 mL of heptane. However, when quantities of used monomers were just 0.4 g, MSP-PEG-COOH yields were reduced to about 50 mg.

To simplify the description of MSP-PEG-COOH prepared under different conditions, the following symbols were utilized throughout. Magnetofluids400 were coated with Mal-PEG-Mal-400 and Mal-PEG-Suc-1540 at a 1:1 molar ratio in a total of 2.0 g; the resulting MSP-PEG-COOH was denoted MSP-PEG-A when the aqueous phase contained sodium carbonate to neutralize half of the carboxyl groups for 18 hours co-polymerization reactions, or MSP-PEG-B when neutral water was used for 6 hours co-polymerization reactions. MSP-PEG-COOH prepared via 6 hours co-polymerization reactions of 2.0 g Mal-PEG-Mal-1540 alone to coat magnetofluids1540 displayed a diameter of about 0.4 μm and was denoted MSP-PEG-D4, and that prepared via 6 hours co-polymerization reactions of 4.0 g Mal-PEG-Mal-1540 alone displayed a diameter of about 0.77 μm and was designated MSP-PEG-D8.

Composition, shapes, and sizes

X-ray diffraction analysis confirmed that magnetofluids and MSP-PEG-COOH contain magnetic cores of Fe_3O_4 (Figure 2). Additionally, the saturation magnetization of MSP-PEG-COOH was reduced after storage in air for three months (see the description later), supporting that Fe_3O_4 is

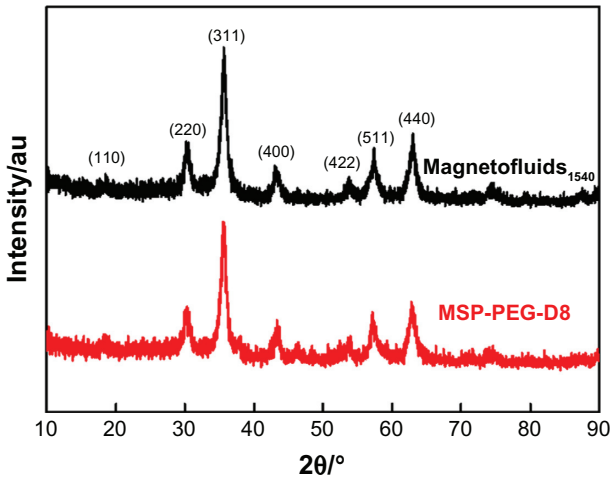

Figure 2 X-ray diffraction analysis of magnetofluids1540 and MSP-PEG-D8.
Abbreviations: MSP, magnetic submicron particle; PEG, poly(ethylene glycol).

the primary magnetic component of such MSPs.[30,31] By FTIR analysis, a characteristic peak at about 1100 cm^{-1} representing vibration of –C–O–C– in PEG was evident in the spectra of MSP-PEG-COOH and Mal-PEG-Mal, but negligible in those of magnetofluids. The characteristic FTIR band at about 630 cm^{-1} for Fe-O was clearly visible for MSP-PEG-COOH and magnetofluids, but absent for PEG derivatives (Figure 3). The small red shift of the FTIR peak for C=O in Mal-PEG-Mal from 1730 cm^{-1} to 1640 cm^{-1} for that in MSP-PEG-COOH indicated changes in the environment of C=O after coating. MSP-PEG-COOH showed greater weight loss in the air atmosphere, compared to the nitrogen atmosphere, and in relation to magnetofluids in either condition (Figure 4A and B). Notably, this weight loss was manifest only at temperatures over 100°C. Hence, MSP-PEG-COOH is a composite particle containing polymerized PEG and the magnetic cores of Fe_3O_4.

The physical appearance of MSP-PEG-COOH was observed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM), and average sizes were estimated by laser scattering. After dispersion via vigorous mechanical stirring for 1 hour in the microemulsion systems without additional monomers, magnetofluids appeared as spheres (magnetic cores) bearing diameters of about 30 nm in TEM pictures under high magnification, and as spheres bearing diameters about 80 nm in SEM pictures (Figure 5). In TEM pictures, MSP-PEG-D8, MSP-PEG-D4, MSP-PEG-A, or MSP-PEG-B appeared as a cluster of multiple nuclei bearing diameters of about 30 nm under high magnification, and as a particle bearing an irregular shape under medium magnification (Figure 5). In SEM pictures, MSP-PEG-D8,

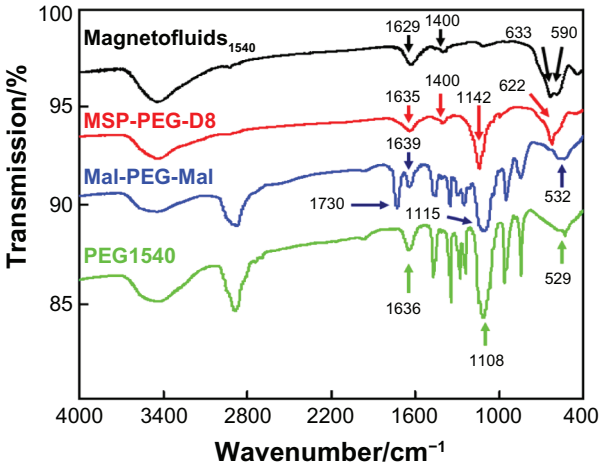

Figure 3 FTIR of PEG-1540, Mal-PEG-Mal-1540, magnetofluids1540, and MSP-PEG-D8.
Abbreviations: FTIR, Fourier transform infrared spectroscopy; PEG, poly(ethylene glycol); MSP, magnetic submicron particle.

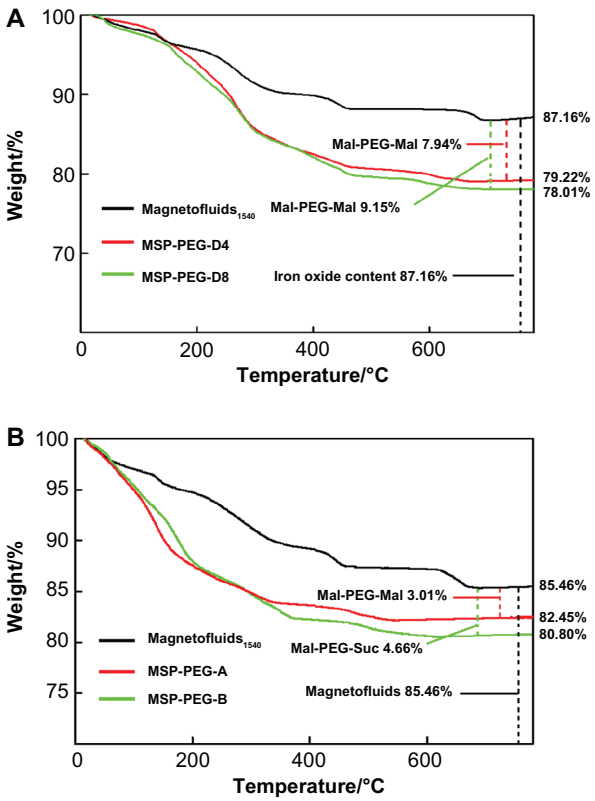

Figure 4 Thermogravimetric analyses. (**A**) magnetofluids1540, MSP-PEG-D4, MSP-PEG-D8; (**B**) magnetofluids 400, MSP-PEG-A, MSP-PEG-B.
Abbreviations: MSP, magnetic submicron particle; PEG, poly(ethylene glycol).

MSP-PEG-D4, MSP-PEG-A, or MSP-PEG-B appeared as an unconsolidated particle and possessed submicron size with an irregular shape similar to that observed by TEM under medium magnification (Figure 5). The shapes and sizes of MSP-PEG-COOH were not affected by vigorous mechanical stirring for several hours in the microemulsion systems. The shapes of MSP-PEG-COOH are similar to those of MSPs from Promega (catalog Z5481 and Z5482; http://cn.promega.com/search-results/?q=Z5481). Furthermore, magnetofluids and MSP-PEG-COOH were suspended in 10 mM of sodium phosphate buffer at pH 7.4 to estimate their average sizes via laser scattering (the buffer was used with all MSP-PEG-COOH, unless otherwise stated). The sizes of the magnetofluids were about 90 nm after dispersion via vigorous mechanical stirring for 1 hour in the microemulsion system, but were much larger before dispersion via vigorous mechanical stirring (Figures 6 and S2). MSP-PEG-D8, MSP-PEG-D4, MSP-PEG-A, and MSP-PEG-B exhibited average sizes of about 0.77 μm, 0.38 μm, 0.32 μm, and 0.34 μm, respectively, and their sizes showed broad distribution (Figure 6 and Table 1). The average sizes of the examined MSP-PEG-COOH were hardly affected by vigorous mechanical stirring for 1 hour

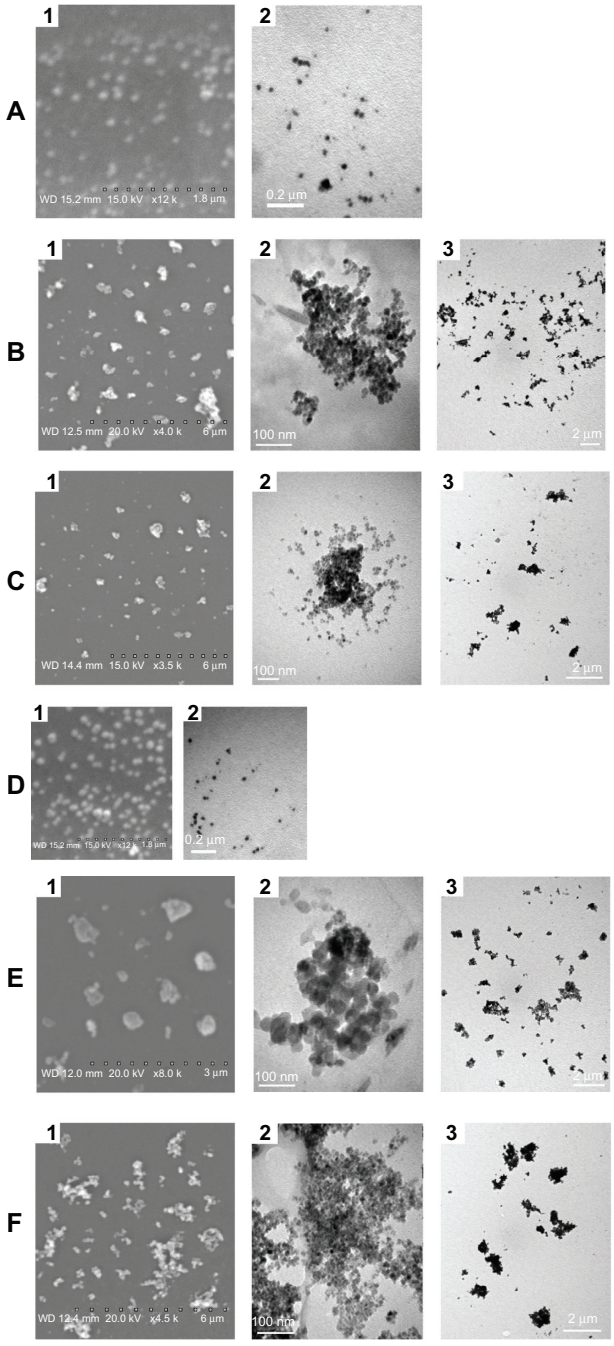

Figure 5 TEM of MSPs. (**A**) Magnetofluids400; (**B**) MSP-PEG-A; (**C**) MSP-PEG-B; (**D**) magnetofluids1540; (**E**) MSP-PEG-D4; (**F**) MSP-PEG-D8. The inserted labels (1), (2), and (3) indicate pictures by SEM, by TEM under high magnification, and by TEM under medium magnification, respectively.
Abbreviations: MSP, magnetic submicron particle; PEG, poly(ethylene glycol); SEM, scanning electron microscopy; TEM, transmission electron microscopy.

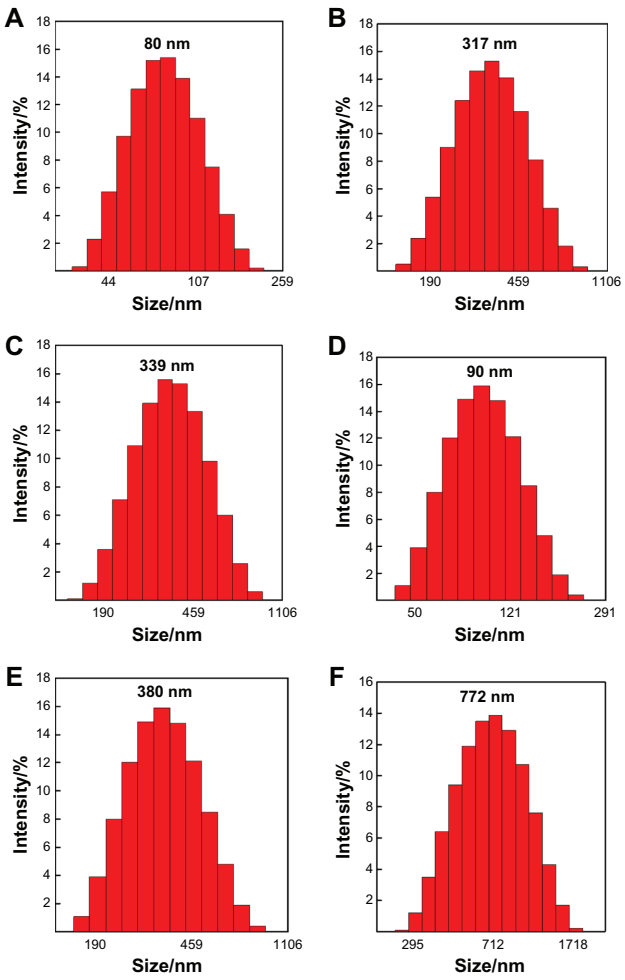

Figure 6 Sizes and distribution via laser-scattering. All sample suspension in the phosphate buffer at pH 7.4 had transmittance of about 30%. Magnetofluids were dispersed via vigorous mechanical stirring in the microemulsion systems before estimation of sizes. (**A**) Magnetofluids400; (**B**) MSP-PEG-A; (**C**) MSP-PEG-B; (**D**) magnetofluids1540; (**E**) MSP-PEG-D4; (**F**) MSP-PEG-D8.
Abbreviations: MSP, magnetic submicron particle; PEG, poly(ethylene glycol).

in the microemulsion systems. Average sizes of MSPs with covalent coats prepared via other approaches were not affected by sonication treatment as well.[32] However, when acrylamide at mass concentrations of 15% or 30% was used for the coating, sphere-like MSPs with sizes over 5 μm were obtained (Figure S3). Therefore, MSP-PEG-COOH prepared by the one-step coating approach with PEG derivatives as monomers have irregular shapes and their sizes and shapes are affected primarily by quantities of monomers used for coating magnetofluids in the microemulsion system.

Taken together, a structural model of MSP-PEG-COOH is depicted (Figure 7); this model implies that there are many accessible carboxyl groups bearing low steric hindrance and this expectation is supported by the experimental results described below.

Suspension and structure stability, saturation magnetization, and nonspecific adsorption

In magnetic separation, MSPs after functionalization with biomolecules should interact with other biomolecules of interest like proteins and nucleic acids in a neutral solu-

Table 1 Summary of properties of tested magnetic materials

Samples/properties	Magnetofluids400	Magnetofluids1540	MSP-PEG-D4	MSP-PEG-D8	MSP-PEG-A	MSP-PEG-B
Size by laser-scattering (μm)	0.36	0.51	0.39	0.77	0.32	0.34
SAV-CIAP binding capacity(mg/g)	nd	nd	1.7	1.1	1.0	2.1
Carboxyl groups (mmol/g)	nd	nd	0.43	0.28	0.13	0.21
Percentage of inorganic material (residual mass at 900°C) (%)	85.5	87.2	79.2	78.0	82.5	80.8
Saturation magnetization (emu/g)	55	57	47	38*	41	46
Saturation magnetization after correction of effects of organic components (emu/g)	65	65	60	49	50	58

Notes: Data from duplicated assays showed coefficient of variation below 12%. *After storage of the sample for 4 months under ambient temperature, its saturation magnetization was reduced to 26 emu/g.
Abbreviations: CIAP, calf intestinal alkaline phosphatase; nd, not determined; MSP, magnetic submicron particle; PEG, poly(ethylene glycol); SAV, streptavidin.

tion within 2 hours.[9,10,15,16,19,33–38] MSPs suitable for such applications should appear as the suspension within this period. All the tested MSP-PEG-COOH displayed favorable suspension stability in 10 mM of sodium phosphate buffer at pH 7.4. MSP-PEG-D4, MSP-PEG-D8, MSP-PEG-A, and MSP-PEG-B produced negligible precipitates, while the magnetofluids displayed evident precipitation in the phosphate buffer at pH 7.4 after incubation for 10 hours at room temperature (Figure S4). Additionally, such MSP-PEG-COOH precipitated rapidly in organic solvents. Few other reported MSPs have such excellent suspension stability in neutral buffers while exhibiting both the same sizes and comparable saturation magnetization (see below). Hence, MSP-PEG-COOH has excellent suspension stability in neutral aqueous solutions, which should be attributed to the PEG chains and abundant carboxyl groups on the coats (see the description later and the structural model in Figure 7).

Any MSP should display sufficient structural stability during the interaction with biomolecules of interest in neutral solutions. All tested MSP-PEG-COOH in the phosphate buffer at pH 7.4 released undetectable quantities of iron ion within 6 hours (Figure S5), and showed stronger resistance to corrosion at pH 10.0 than pH 7.4, but weaker resistance at pH 4.0. Moreover, MSP-PEG-A and MSP-PEG-B displayed slightly stronger resistance to corrosion at pH 4.0 than MSP-PEG-D4 and MSP-PEG-D8. After storage for 6 hours in 0.20 M of sodium acetate buffer at pH 4.0 and magnetic separation, supernatant fractions of MSP-PEG-COOH were nearly transparent, while those of the magnetofluids were cloudy (Figure S6). Stronger resistance of MSP-PEG-COOH to corrosion in (slightly) alkaline aqueous buffers than magnetofluids is attributed to the protective effects of their covalent coats, and supports that MSP-PEG-COOH can be utilized in slightly alkaline solutions and even be stored in (slightly) alkaline solutions.

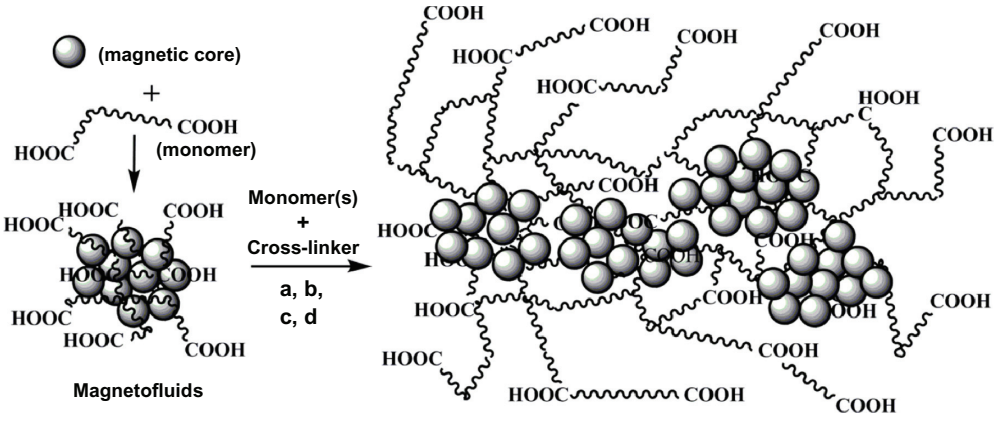

Figure 7 Coating of magnetofluids to prepare MSP-PEG-COOH. (**a**) Mixing 0.70 mL of magnetofluids in 3.0 mL of water, 5.0 mL aqueous solution of 2.0 or 4.0 g monomer(s), and 1.0 mL of cross-linker N,N′-methylene bis-acrylamide saturated at 25°C to make about 10 mL of aqueous phase. (**b**) Mixing 500 mL of heptane and 12 g of AOT to make a solution for dispersing the aqueous phase and making the microemulsion system. (**c**) Addition of ammonium peroxydisulfate saturated at 25°C in 1.0 mL of water. (**d**) Addition of 1.0 mL of 0.5% solution of N,N,N′,N′-tetramethylethylenediamine.
Abbreviations: MSP, magnetic submicron particle; PEG, poly(ethylene glycol); AOT, aerosol-OT.

MSPs for biomedical applications are preferred to have the maximal possible saturation magnetizations. Saturation magnetizations of MSP-PEG-B and MSP-PEG-D4 were about 46 emu/g, accounting for about 80% of the values for the magnetofluids, and were higher than those of MSP-PEG-A and MSP-PEG-D8, respectively (Figure 8 and Table 1). Such saturation magnetizations of MSP-PEG-B and MSP-PEG-D4 are favorable over those of most MSPs prepared via the classical approaches.[1–8] TGA revealed that MSP-PEG-A and MSP-PEG-B displayed smaller loss of weight than MSP-PEG-D4 and MSP-PEG-D8 (Figure 4). Thus, types and quantities of monomers for the coating of magnetofluids primarily affect saturation magnetization of the resulting MSPs; for higher saturation magnetizations of MSPs, it is favorable to use a longer linear hydrophilic monomer of single polymerizable bond but two carboxyl groups at two separate ends plus shorter linear hydrophilic monomer(s) of two polymerizable bonds, or a smaller quantity of a reasonably long linear hydrophilic monomer bearing two polymerizable bonds at two separate ends, for the coating of magnetofluids in the microemulsion system.

MSPs suitable for magnetic separation should exhibit negligible nonspecific interactions; this prerequisite can hardly be directly satisfied by MSPs prepared via common classical approaches.[1–8] Adsorption of hydrophobic substances on MSPs reflects their tendency to exert nonspecific interactions with common substances. A number of aromatic compounds with logP from −1.8 to about 5.7 were examined for nonspecific adsorption on MSP-PEG-D4 and MSP-PEG-B. 4-Nitronaphthyl-1-octyl ester with logP of about 5.7 displayed significant nonspecific adsorption, while the other compounds with logP values below 4.0 exhibited undetectable nonspecific adsorption at concentrations of no more than 20 μM (Figure S7). There may be the multiple-layer adsorption/accumulation of the long flexible 4-nitronaphthyl-1-octyl ester in the loose outer surface of MSP-PEG-COOH (Figure 7). Slow injection of neutralized MSP-PEG-D4 into rabbit caused negligible stimulation and no acute inflammation response (data not given). Hence, MSP-PEG-D4 and MSP-PEG-B exhibit negligible nonspecific interactions with compounds of small size and low or medium hydrophobicity; they may be applicable to immobilize target proteins for screening mixture-based ligand libraries.

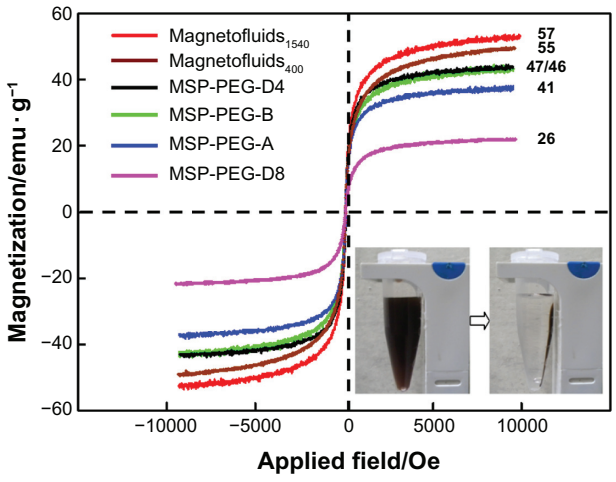

Figure 8 Saturation magnetizations of magnetofluids and MSP-PEG-COOH.
Abbreviations: MSP, magnetic submicron particle; PEG, poly(ethylene glycol).

Taken together, the one-step coating approach produces MSPs that concurrently exhibit excellent suspension stability, good chemical stability, high saturation magnetization, and negligible nonspecific adsorption of common substances; these features imply the incomparable advantages of the one-step coating approach over other classical ones for preparing MSPs.

Binding capacity of immobilized biotin

For magnetic separation, MSP-PEG-COOH should be functionalized with biomolecules to exert specific interactions with the counterparts in solutions. SAV can form tight complexes with biotin moiety as long as they are accessible to each other.[9,10] Carboxyl groups on MSPs can be quantified via titration against a NaOH solution and completely conjugated with biotin after activation into active esters. Namely, saturated immobilization of biotin on these carboxyl groups of MSPs can be achieved. The binding capacity for SAV of a unit quantity of biotinylated MSPs is a direct index of the accessibility to SAV and the specific binding capacity of immobilized biotin, as well as an indirect reflection of steric hindrance around carboxyl groups that are covalently conjugated to biotin. With the conjugate of SAV and alkaline phosphatase (SAV-CIAP) as the probe, the quantity of bound SAV can be easily estimated based on the activity of alkaline phosphatase. Hence, the binding capacity for SAV was tested with SAV-CIAP.

For MSP-PEG-COOH prepared with the tested Mal-PEG-Mal, the average quantities of titrated carboxyl groups were over 0.11 mmol/g (Table 1 and Figure S8). Quantities of carboxyl groups on MSP-PEG-D4, MSP-PEG-D8, MSP-PEG-B, and MSP-PEG-A were approximately 0.43, 0.28, 0.21, and 0.13 mmol/g, respectively. Moreover, MSP-PEG-COOH prepared with PEG derivatives as monomers bearing two carboxyl groups had a quantity of carboxyl groups 10-fold higher than that of MSPs prepared with a single-ended maleic monoester of PEG of 1540 Da.[26] On the other

hand, after saturated immobilization of biotin, MSP-PEG-D8 and MSP-PEG-A displayed the binding capacities of about 1.0 mg/g for SAV-CIAP, while MSP-PEG-D4 and MSP-PEG-B exhibited the binding capacities of about 1.7 and 2.1 mg/g for SAV-CIAP, respectively (Table 1 and Figure S9). Notably, the use of Mal-PEG-Mal-400, Mal-PEG-Mal-4000, or Mal-PEG-Mal-6000 as the unique monomer for coating magnetofluids led to a reduction in the binding capacity of biotinylated MSPs for SAV-CIAP (Table S1). Assuming one biotin was conjugated to one titrated carboxyl group, the specific binding capacity for SAV of biotin immobilized on MSP-PEG-B was comparable to that on MSP-PEG-A, over twice that on MSP-PEG-D4 or MSP-PEG-D8, and nearly 100 times higher than that on MSPs prepared with a single-ended maleic monoester of PEG of 1540 Da.[26] These results support that the use of a longer monomer bearing just one double bond for polymerization during coating alleviates the cross-linking of monomers on surfaces of MSPs and thus steric hindrance around carboxyl groups (Figure 9); MSP-PEG-B and MSP-PEG-D4 after functionalization with biotin have sufficient binding capacities for SAV conjugates.

On the other hand, the screening of ligands of high affinity in mixture samples is most absorbing for drug discovery in terms of cost and efficiency, but requires magnetic separation of target-ligand complexes.[9,10,33–38] Target proteins are usually produced by recombinant expression via fusion to special tag proteins like glutathione-S-tranferase and maltose-binding-protein;[39,40] or else, native target proteins after purification can be conjugated with SAV that serves as a tag. In this case, a ligand bearing extremely strong affinity to a tag fused or conjugated to target proteins can be immobilized on MSP-PEG-B or MSP-PEG-D4 for magnetic separation of target-ligand complexes. Clearly, the conjugates of biotin and MSP-PEG-B or MSP-PEG-D4 can be suitable biomaterials for magnetic separation of target-ligand complexes as long as target proteins can be facilely conjugated to SAV with negligible alteration on the function of either one.

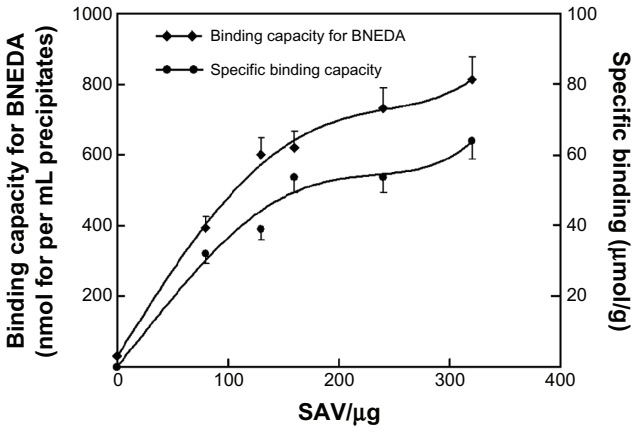

Figure 9 Immobilization of SAV on MSP-PEG-D4.

Notes: Carboxyl groups on MSP-PEG-D4 were activated as NHS ester in THF. Each mixture in 1.0 mL for immobilization reaction contained an indicated quantity of SAV in 0.60 mL of the same phosphate buffer, and 0.40 mL suspension of MSP-PEG-D4 after 1:10 dilution of the packed precipitates with cold 10 mM sodium phosphate buffer at pH 8.0. Binding capacity was estimated with BNEDA as the probe and calculated for the unit volume of compacted precipitates of the conjugates. Residual SAV in the supernatant was quantified as described before.[37] Specific binding was calculated for per gram of SAV immobilized on MSP-PEG-D4. Results were from assays in triplicate with CV below 20%.

Abbreviations: BNEDA, N-(biotinyl)-N′-(1-naphthyl)-ethylenediamine; CV. coefficient of variation; MSP, magnetic submicron particle; NHS, N-hydroxysuccinimide; PEG, poly(ethylene glycol); SAV, streptavidin; THF, tetrahydrofuran.

Immobilization of streptavidin

The conjugates of SAV and MSPs are widely utilized for bioaffinity separation, detection and indirect immobilization of biotinylated biomolecules,[19,20,33–35,38] and are pivotal biomaterials in reagent kits for automated chemiluminescence immunoassays in clinical laboratories.[41–43] Hence, we examined the immobilization of SAV on MSP-PEG-COOH via N-acylation.

MSP-PEG-D4 after the activation of carboxyl groups immobilized SAV at about 10 mg/g MSPs via N-acylation (Figure 9). The specific activity of immobilized SAV for BNEDA, a small fluorescent biotin derivative as the probe, was 81% ± 18% (n = 5) of that of free SAV. However, glutathione-S-transferase is sensitive to modification of its amino groups; glutathione-S-transferase immobilized on MSP-PEG-D4 via N-acylation retained only about 5% of its original activity (data not given). The high activity of immobilized SAV is attributed to resistance of SAV to N-acylation. The maximal binding capacity of the conjugates of SAV and MSP-PEG-D4 for the small probe was about 700 nmol for per mL precipitates of MSPs (about 3.6 μmol/g of MSPs), comparable to those of commercial products bearing similar physical shapes.[9] Protein targets and other biomolecules can be easily modified with biotin. Hence, the conjugates of SAV and MSP-PEG-D4 appear suitable for magnetic separation of biotinylated molecules in mixtures, and thus may be applicable to magnetic separation for the screening of ligands in mixture samples and chemiluminescence immunoassay.

Conclusion

The following conclusions are drawn: (1) One-step coating of magnetofluids via radical polymerization of unsaturated carboxylic derivatives of PEG in water-in-oil microemulsion systems yields MSP-PEG-COOH concomitantly bearing many properties favorable for magnetic separations;

(2) the one-step approach is potentially applicable to coat common inorganic cores for producing coated particles with many favorable properties for biomedical applications; (3) structures, concentrations, and compositions of hydrophilic monomers, the quantities of water, and AOT in the microemulsion systems, should be further optimized to improve shapes, structural stability, sizes, and size distribution of MSPs.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

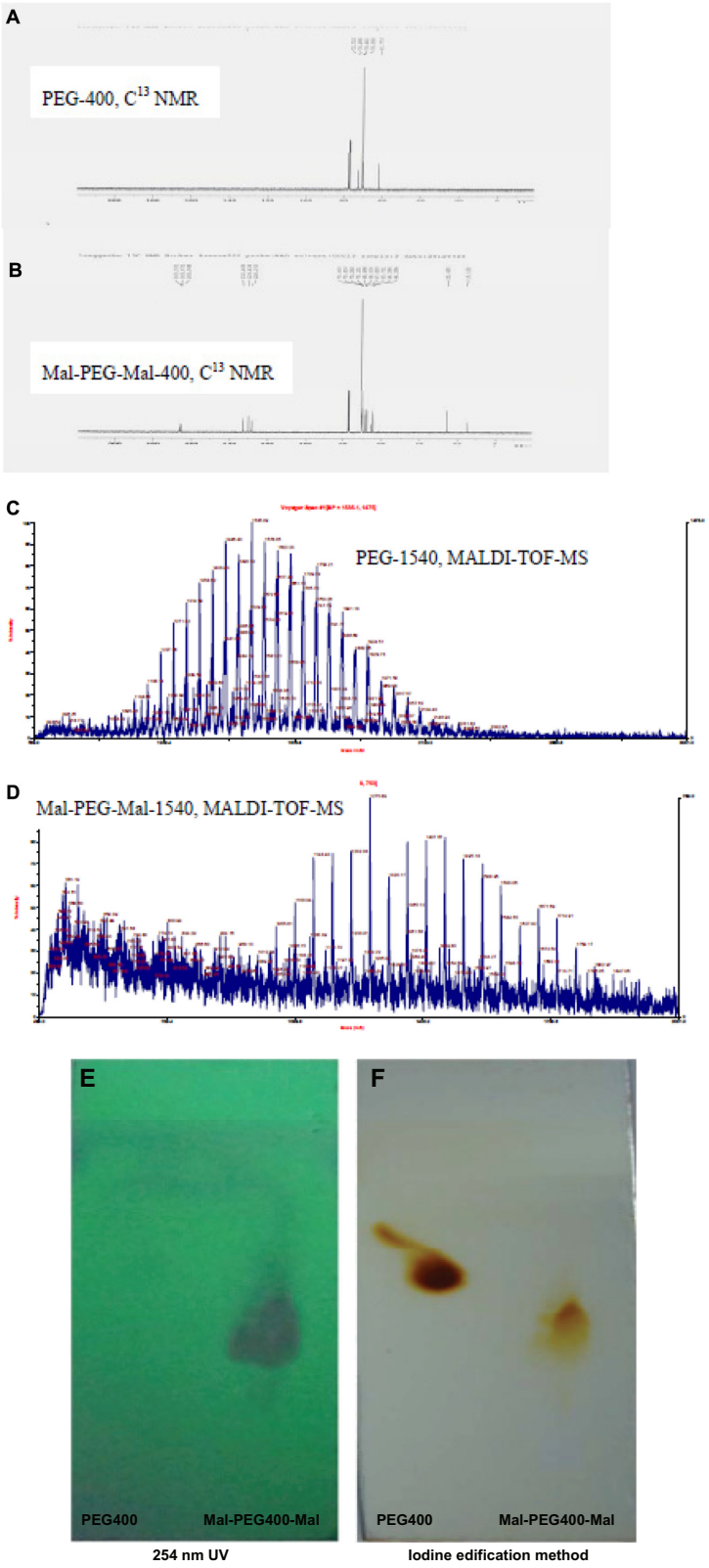


Figure S1 Data to examine the structures of Mal-PEG-Mal-400, Mal-PEG-Mal-1540. (**A**) PEG-400, C^{13} NMR; (**B**) Mal-PEG-Mal-400, C^{13} NMR; (**C**) PEG-1540, MALDI-TOF-MS; (**D**) Mal-PEG-Mal-1540, MALDI-TOF-MS; (**E**) 254 nm UV; (**F**) iodine edification method.

Abbreviations: C^{13} NMR, carbon-13 nuclear magnetic resonance; Mal-PEG-Mal, PEG-bis-maleic monoester; PEG, poly(ethylene glycol); UV, ultraviolet.

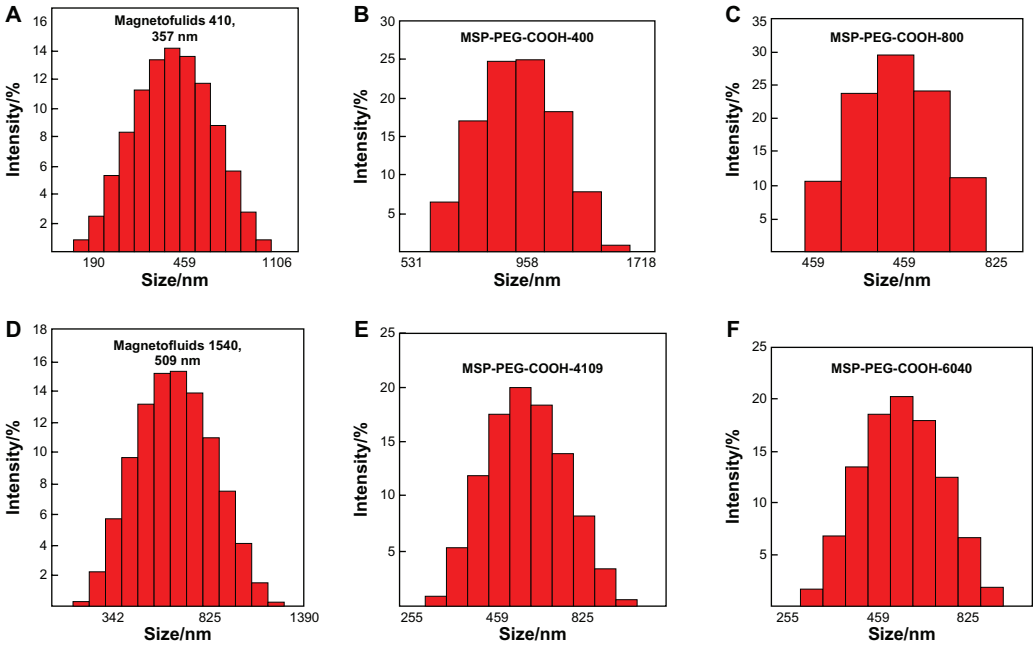


Figure S2 Size distribution of MSPs estimated by laser scattering. Samples were suspension with transmittance of about 30% in the phosphate buffer at pH 7.4. (**A**) Magegnetofluids 400; (**B**) MSP-PEG-D4; (**C**) MSP-PEG-D8; (**D**) magnetofluids 1540; (**E**) MSP-PEG-COOH-4109; (**F**) MSP-PEG-COOH-6040.
Abbreviations: PEG, poly(ethylene glycol); MSP, magnetic submicron particle.

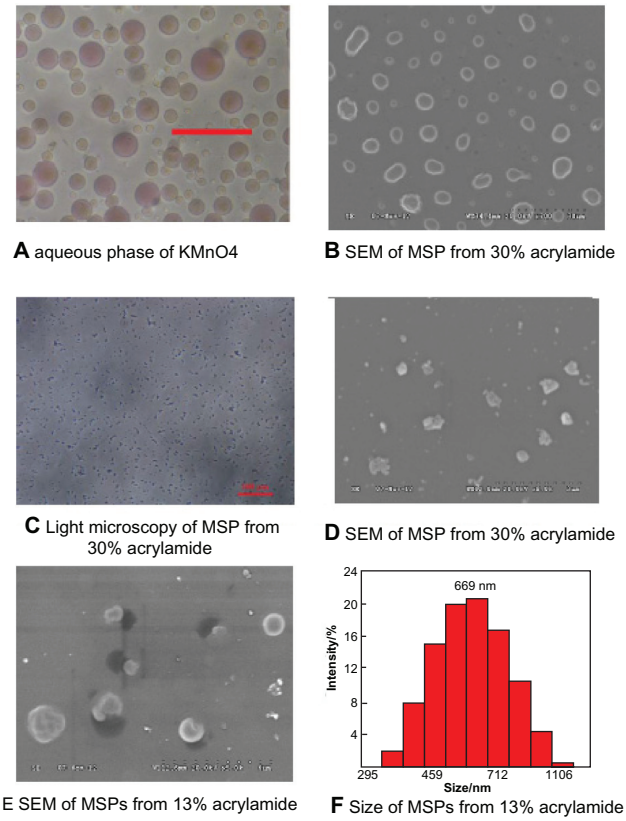


Figure S3 Examination of MSPs coated with polyacrylamide and sizes of aqueous phases. (**A**) Aqueous phase of KMn04; (**B**) SEM of MSP from 30% acrylamide; (**C**) light microscopy of MSP from 30% acrylamide; (**D**) SEM of MSP from 13% acrylamide; (**E**) SEM of MSPs from 13% acrylamide; (**F**) Size of MSPs from 13% acrylamide.
Abbreviations: MSP, magnetic submicron particle; SEM, scanning electron microscopy.

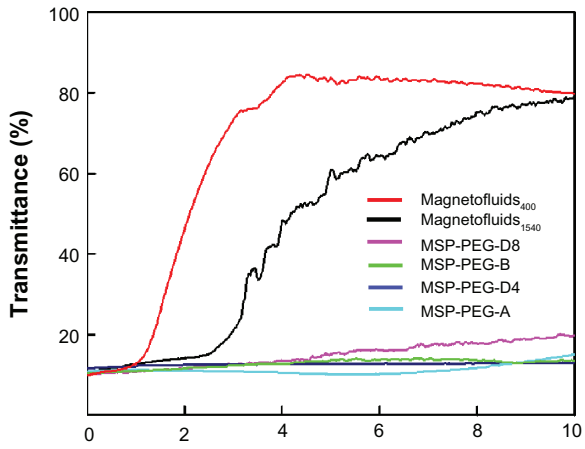


Figure S4 Suspension stability of some representative MSPs.
Abbreviations: MSP, magnetic submicron particle; PEG, poly(ethylene glycol).

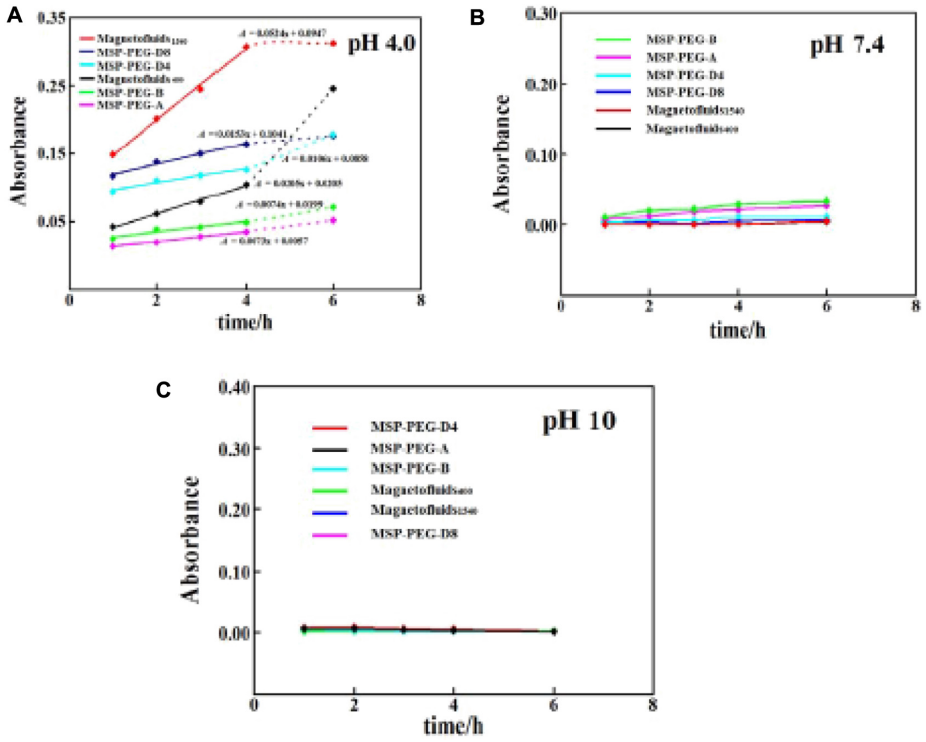
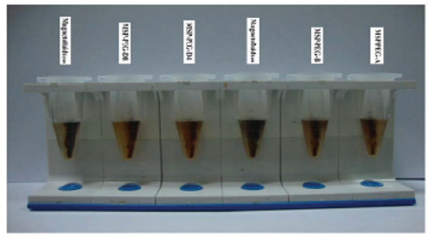
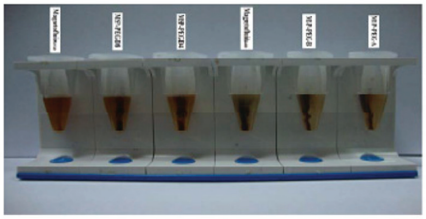


Figure S5 Resistance to corrosion by buffers at different pH. (**A**) pH 4.0; (**B**) pH 7.4; (**C**) pH 10. See context for details of operation. CV was usually 5% for assays in triplicate.

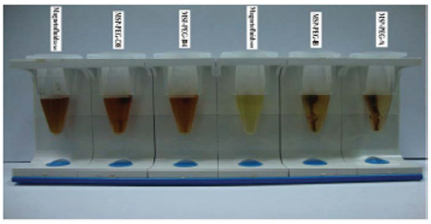
Abbreviations: CV. coefficient of variation; MSP, magnetic submicron particle; PEG, poly(ethylene glycol).



Sodium acetate buffer (0.2 M) at pH 4.0/0 h. 22°C



Sodium acetate buffer (0.2 M) at pH 4.0/4 h. 22°C



Sodium acetate buffer (0.2 M) at pH 4.0/28 h. 22°C

Figure S6 Photographic records of resistance of magnetic materials to acid corrosion. Sodium acetate buffer (0.2 M) at pH 4.0, 22°C for (**A**) 0 hours; (**B**) 4 hours; (**C**) 28 hours.

Abbreviations: MSP, magnetic submicron particle; PEG, poly(ethylene glycol).

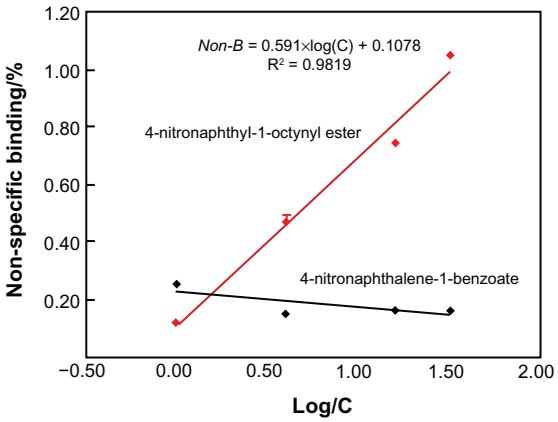


Figure S7 Nonspecific bindings of compounds of stronger hydrophobicity.

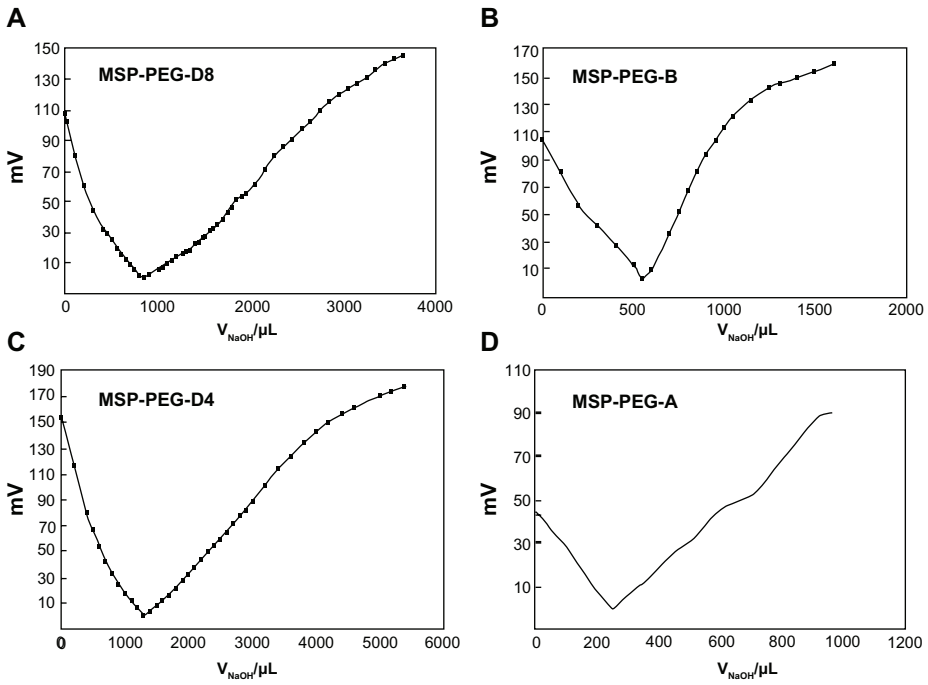


Figure S8 Titration curves of carboxyl groups on MSPs. (**A**) MSP-PEG-D8; (**B**) MSP-PEG-B; (**C**) MSP-PEG-D4; (**D**) MSP-PEG-A.
Abbreviations: MSP, magnetic submicron particle; PEG, poly(ethylene glycol).

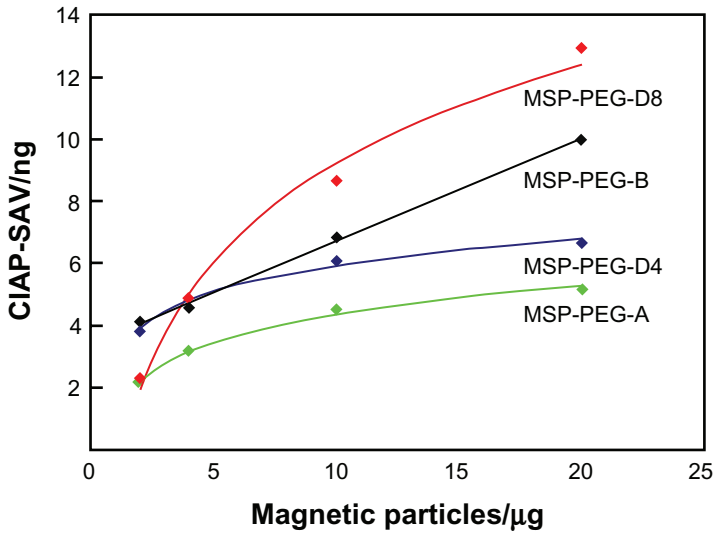


Figure S9 Binding capacity of biotin immobilized on MSPs.
Abbreviations: CIAP. calf-intestinal alkaline phosphatase; MSP, magnetic submicron particle; PEG, poly(ethylene glycol); SAV. streptavidin.

Table S1 Summary of properties of tested magnetic materials

Samples/properties	MSP-PEG-COOH prepared with Mal-PEG-Mal-400	MSP-PEG-COOH prepared with Mal-PEG-Mal-800	MSP-PEG-COOH prepared with Mal-PEG-Mal-4000	MSP-PEG-COOH prepared with Mal-PEG-Mal-6000
Size by laser-scattering (μm)	0.89	0.69	0.55	0.51
SAV-CIAP binding capacity($mg \cdot g^{-1}$)	0.02	0.30	0.55	0.50
Carboxyl groups ($mmole \cdot g^{-1}$)	0.25	0.23	0.19	0.16

Notes: Sizes and quantities of carboxyl groups showed coefficient of variation below 12% from duplicated assays. Just one monomer at 4.0 g was used in each microemulsion system.
Abbreviations: CIAP, calf intestinal alkaline phosphatase; MSP, magnetic submicron particle; PEG, poly(ethylene glycol); SAV, streptavidin.