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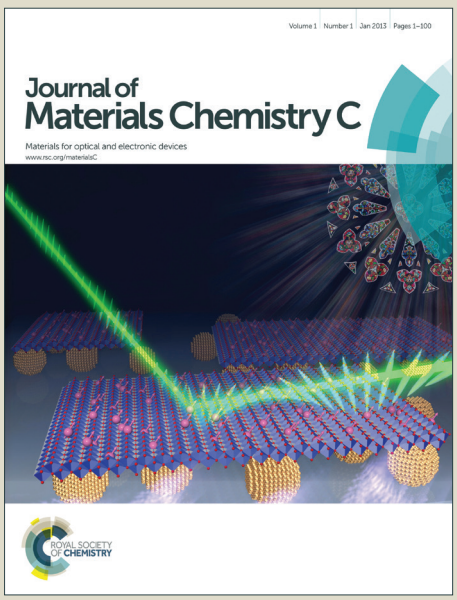

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ARTICLE

Rewritable Magnetic Fluorescent-Encoded Microspheres: Preparation, Characterization, and Recycling

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Rewritable magnetic fluorescent-encoded microspheres were prepared by coating magnetic microspheres with fluorescent-encoded melamine-formaldehyde (MF) shell. Fluorescent encoding was realized by varying the dye types and concentrations incorporated into the MF shell. Ten sets of magnetic fluorescent-encoded microspheres including four sets doped with single dye and six sets doped with two dyes were obtained and successfully identified through flow cytometric analysis. High encoding capacity could be achieved by increasing the dye number and intensity levels. More importantly, the original fluorescent-encoded MF shell could be removed via calcination. The inner magnetic microspheres remained unaffected and could be re-coated with new fluorescent-encoded MF shell easily. The fluorescent MF coating (encoding writing) and calcination (encoding erasing) composed a cycle. Results demonstrated that the magnetic microspheres maintained their original characteristics after writing-erasing cycles and the writing-erasing cycle could be repeated numerous times. This kind of rewritable magnetic fluorescent-encoded microspheres enabled recycling and regeneration. Combining magnetic separation and re-encoding which can significantly simplify the analysis processes and decrease the analysis cost, these microspheres are expected to be widely used in suspension array for high-throughput analyses.

Introduction

Magnetic microspheres are widely used in various applications, such as targeted drug delivery,[1] magnetic resonance imaging,[2] hyperthermia,[3] biosensing,[4] and magnetic separation.[5] With magnetic microspheres as carriers, magnetic separation has been extensively employed in water treatment,[6] enzyme and catalyst immobilization,[7, 8] nucleic acid isolation,[9] cell labeling and sorting,[10, 11] and protein separation.[12-15] Magnetic separation has several advantages. First, this process is time and labor saving. Magnetic microspheres can be conveniently manipulated by external magnetic fields, thereby simplifying separation and recovery. Second, magnetic separation is applicable to crude samples without the need of pretreatment, such as centrifugation or filtration. Third, magnetic separation is gentle to biomolecules, hence avoiding the shear forces caused by conventional centrifugation. Fourth, magnetic separation is easily miniaturized and automated. Given these advantages, magnetic separation has more potential for high-throughput sample analyses and is easily to realize integrated and miniaturized analyses compared with conventional separation technologies. Automated magnetic separators are commercially available, which enable the isolation and purification of 96 samples simultaneously.[16] Magnetic microspheres are also applied in microfluidic devices, including fluid mixing, analyte capture, concentration and transfer, and magnetic field-assisted washing steps.[17]

The fast-increasing clinical diagnoses, environmental pollution analyses, and biochemical studies require rapid and simultaneous analyses for a large number of samples. Combining magnetic separation and high-throughput analysis technology will greatly simplify the analysis process and realize automatization and integration, which will greatly reduce the manual labor and analysis time.[18, 19] Suspension array is a promising platform for multiplex analyses of biomarkers, food and environmental monitoring, and drug screening.[20] In contrast to conventional planar microarray that uses chip spatial positions to label targeted molecules, suspension array employs encoded microspheres coupled with specific receptor molecules as array elements for rapid target identification.[18] Encoded microspheres can be prepared in bulk with large coding capacities, whereas the number of planar microarray prepared at one time is limited. With encoded microspheres being the reaction carriers and encoding elements, suspension array shows advantages in high-throughput analyses, flexible array preparation, faster binding kinetics, and high reproducibility.[21] Among various encoding patterns, such as graphical encoding, photonic crystal reflection spectrum encoding, and Raman spectrum encoding, fluorescence spectrum encoding has attracted considerable attention as it offers many advantages in terms of simple operation (encoding and decoding), high sensitivity, and high encoding

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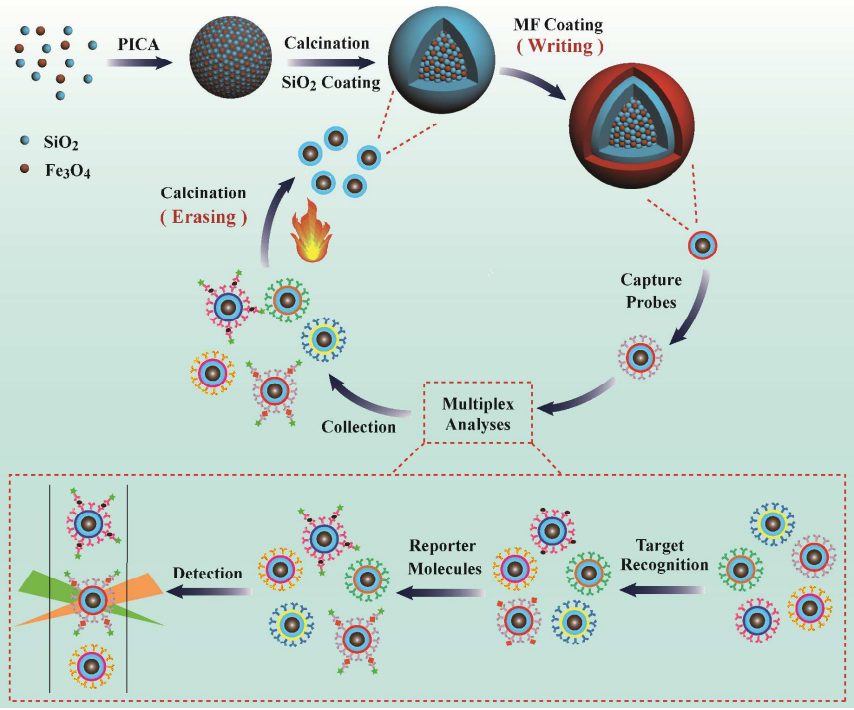
capacity. Integrating magnetic microspheres and fluorescent-encoded microspheres will combine the merits of magnetic separation and multiplex analyses. Compared with non-magnetic microspheres, magnetic fluorescent-encoded microspheres possess several advantages.[19] Magnetic fluorescent-encoded microspheres can be easily separated from complex mixtures, thereby enabling colloidal or particulate sample analysis without pretreatment.[22] Magnetic responsiveness also confers the analysis with magnetic enrichment, which further increases the detection sensitivity. Given the fact that magnetic separation can be automated and miniaturized, magnetic fluorescent-encoded microspheres can be integrated with microfluidic devices for low-cost biochemical analyses and point-of-care medical diagnoses.[23]

Magnetic responsiveness enables easy and automated separation, but recycling the magnetic fluorescent-encoded microspheres remains difficult as different encoded microspheres are mixed together to realize multiplex analyses. Considering the high preparation costs and large amount of microspheres that are potentially used in multiplex analyses, microspheres should be recycled to prevent significant waste. However, to the best of our knowledge, studies investigating the recycling and regeneration of magnetic fluorescent-encoded microspheres are limited. From our point of view, two possible solutions might be used to recycle the microspheres. One is recycling the microspheres separately according to their own encoding information, which requires complicated sorting equipment to collect the microspheres. Meanwhile, the bound analytes must be removed completely, otherwise the residual analytes will interfere with the later experiments. These requirements make separate recycling very difficult, even impractical. The other possible solution is to recycle the microsphere mixtures and re-encode the microspheres, thus enabling the different encoding of the same microspheres and multiple recycled uses. This method requires simple and low-cost procedures to remove the encoding information and conjugated receptor molecules completely. Unfortunately, there were barely reports concerning these problems have been found.

Given the results described above, we prepared recyclable and reproducible magnetic fluorescent-encoded microspheres based on our previous reports. (1) We previously reported the preparation of fluorescent-encoded melamine-formaldehyde (MF) microspheres by quantitatively incorporating various dyes during the microsphere preparation.[24] The fluorescent-encoded MF microspheres were easily prepared using relatively cheap materials and with good thermal and mechanical stabilities. High encoding capacity was achieved by adjusting the dye species and doping concentrations. (2) We also reported the preparation of narrow-dispersed magnetic microspheres in our previous work.[25] To obtain a high saturation magnetization, calcination was conducted to remove the polymer resins in the microspheres with inorganic materials maintained. In the present work, magnetic fluorescent-encoded microspheres were prepared by coating the magnetic microspheres with fluorescent-encoded MF shell. Similarly, the fluorescent-encoded MF shells could be removed by calcination owing to their organic composition. The inner magnetic microspheres remained unchanged; therefore, the fluorescent-encoded MF shell coating could be performed again. This process can be repeated several times, and "rewritable" magnetic fluorescent-encoded microspheres will be realized (Scheme 1). Magnetic urea-formaldehyde microspheres were initially prepared by polymerization-induced colloid aggregation method (PICA). Silica sol was added to reduce the light absorption of the magnetic microspheres and enhance the mechanical properties simultaneously.[26] Magnetic core/silica shell (MS) microspheres were obtained after the removal of polymer resin via calcination followed by a further silica coating. The silica layer separated the magnetic core and fluorescent shell, thus eliminating the fluorescence quenching of the magnetic core. Magnetic fluorescent-encoded microspheres were obtained by coating the MS microspheres with fluorescent-encoded MF shell (writing of encoding information), hereinafter referred to as MS-MF microspheres. After conjugation of capture probes, the MS-MF microspheres could be used for magnetic-assisted multiplex analyses. The red dashed box in scheme 1 illustrates the procedures for encoded microsphere-based multiplex analyses. Different encoded microspheres each conjugated with specific capture probes were incubated with the samples. The targets firstly bound with the capture probes and further bound to fluorescence labeled reporter molecules. The encoding information of encoded microspheres was used to identify the target molecules whereas the reporter molecules were used for quantification. The mixtures of different fluorescent-encoded microspheres after analysis could be collected conveniently via magnetic separation. The fluorescent MF shell and bound biomolecules could be easily and simultaneously removed via calcination in air (erasing of encoding information), while keeping the inner MS microspheres unaffected. The MS microspheres after calcination could be used to prepare the magnetic fluorescent-encoded microspheres again. The writing and erasing processes comprise a cycle. Our experiments proved that the MS microspheres with different cycles had similar properties, and all could be repeatedly subjected to the writing process. Combining the magnetic separation and rewritable characteristics, this kind of MS-MF microspheres could be encoded with different fluorescence information (dye type and concentration) and be utilized many times, thus would be of great significance in simplifying the analysis processes and reducing the analysis costs. The recycling and regeneration method in the present paper was rather simple and low-cost as no other reagents were involved. Besides, the recycled MS microspheres were successfully modified with polydopamine coating, thus could also be used in various applications,[27] such as enzyme immobilization, nucleic acid extraction, and magnetic catalyst carrier.

Experimental Section

Materials



Scheme 1. Preparation and erasing-writing cycle of magnetic fluorescent-encoded microspheres.

Formaldehyde (37 wt%), urea, ammonia (25 wt%), nitric acid (65 wt%), hydrochloric acid (37 wt%), and ethanol were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Silica sol (30% SiO_2, 10-15 nm) was obtained from Guolian Technology Co., Ltd. (Jiangyin, China). Fe_3O_4 nanoparticles (20 nm), tetraethoxysilane (TEOS, 98%), melamine, paraformaldehyde, hydrochloric acid standard solution (1.0 mol·L^{-1}), Rhodamine 110 chloride (Rh 110), and glutaraldehyde (GA, 50 wt%) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Sulforhodamine 101 (SRh 101), bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO) were acquired from Sigma-Aldrich (St. Louis, MO, USA). All reagents were of analytical grade and used without further purification. The BCA Protein Assay Kit was obtained from Beyotime Biotechnology (Jiangsu, China). Mouse immunoglobulin G (IgG), goat anti-mouse IgG, FITC-goat anti-mouse IgG were obtained from Elabscience Biotechnology Co., Ltd. (Wuhan, China). Double distilled water was used in all experiments.

Preparation of magnetic microspheres

Water-based ferrofluids were prepared according to our former report without concentration.[25] Magnetic urea-formaldehyde microspheres were prepared through PICA with a slight modification. About 100 mL of ferrofluids, 5 mL of silica sol (pH 2), 8 mL of urea solution (200 mg·mL^{-1}, pH 2), and 4 mL of formaldehyde solution were mixed thoroughly and heated at 60 °C for 30 min. The magnetic urea-formaldehyde microspheres were washed with distilled water and dried at 60 °C. Magnetic microspheres were obtained via calcination in air to remove the urea-formaldehyde resin. Calcination was carried out by heating the microspheres from 50 °C to 600 °C with a temperature ramp of 5 °C·min^{-1}, maintaining at 600 °C for 1 h, and cooling to room temperature naturally.

Preparation of MS microspheres

Approximately 0.1 g of the obtained magnetic microspheres was dispersed in the solution containing 10 mL of distilled water, 40 mL of ethanol, and 1 mL of ammonia. About 0.15 mL of TEOS was added dropwise under stirring to the mixture. The reaction was continued for 3 h. The coating process was repeated once to ensure that the silica shell was sufficiently thick. Ultrasonication was conducted during the coating process to prevent agglomeration of magnetic microspheres. The magnetic microspheres with silica coating were washed with ethanol and dried at 60 °C. The same calcination process was conducted to obtain the final magnetic core/silica shell MS microspheres, which were dispersed in distilled water with a concentration of 100 mg·mL^{-1} and stored at 4 °C.

Preparation of MS-MF microspheres

The preparation of MF resin pre-polymer solution and dye doping process were conducted according to our former report with a slight modification.[24] About 2.6 g of melamine, 3.7 g of paraformaldehyde, and 100 mL of distilled water were mixed and heated at 60 °C for 40 min. Rh 110 and SRh 101 were used as representative dyes. These dyes were dissolved in DMSO at a concentration of 10 mmol·L^{-1} as stock solution. Dye-doped MF resin pre-polymer solutions were obtained by mixing a certain volume of dye stock solutions with the MF resin pre-polymer solutions. 10 mg of the MS microspheres were mixed with 5 mL of 4×10^{-3} mol·L^{-1} diluted hydrochloric acid and 0.6 mL of the dye-doped MF resin pre-polymer solution. The mixture was heated in a boiling water bath for 15

min. Different fluorescent encodings were obtained by adjusting the dye species and concentrations in the pre-polymer solution. The magnetic fluorescent-encoded MS-MF microspheres were washed with distilled water and stored at 4 °C for further use.

Characterization

Phenom G2 Pro scanning electron microscopy (SEM) (New York, USA) was used to characterize the microsphere morphologies. Fourier transform infrared (FTIR) spectra were recorded on a Bruker Optics Tensor spectrometer (Ettlingen, Germany). Magnetic hysteresis loops were tested on a Lake Shore 7410 vibrating sample magnetometer (Maryland, USA) at room temperature. Zeta potential measurements were performed using Malvern Zetasizer Nano ZS90 (Worcestershire, UK). Laser scanning confocal microscopy (LSCM) images were captured using a Carl Zeiss LSM 700 laser scanning confocal microscope (Oberkochen, Germany). Fluorescence spectra were recorded using a HORIBA Jobin Yvon FluoroMax-4 fluorescence spectrophotometer (Kyoto, Japan). Flow cytometric analysis was performed on a BD FACSCanto™ II flow cytometer (New Jersey, USA) with 488 nm excitation.

Protein conjugation and sandwich immunoassay

The surface protein conjugation of the MS-MF microspheres was conducted through electrostatic adsorption and covalent attachment.[28, 29] BSA molecules were firstly adsorbed on the positive surface of MS-MF microspheres by dispersing the microspheres in 2 mg·mL^{-1} BSA solution (0.5 M NaCl) for 30 min. Subsequently, the microspheres were washed thrice with PBS containing 0.01% Tween 20, dispersed in 0.05% GA solution (PBS diluted, 0.01% Tween 20) for 12 h, and washed with PBS (0.01% Tween 20). The microspheres were modified with reactive aldehyde groups, which could readily react with the amine groups of many molecules. We used BSA as a model molecule to investigate the surface protein conjugation. The GA-modified microspheres were dispersed in 1 mg·mL^{-1} BSA solution (PBS, 0.01% Tween 20) for 12 h. The amount of conjugated BSA was determined according to the protocol of the BCA Protein Assay Kit.

Goat anti-mouse IgG was also covalently attached to the MS-MF microspheres through reaction with aldehyde groups. 1 mg of GA-modified microspheres were dispersed in 200 μL PBS (0.01% Tween 20), and 20 μL of goat anti-mouse IgG solution was added. After incubating at room temperature for 5h, the goat anti-mouse IgG-bearing MS-MF microspheres were washed three times with PBS buffer (0.01% Tween 20) and blocked with 400 μL of BSA solution (1%, PBS) overnight at 4 °C. For IgG group, 20 μL of mouse IgG was added to the mixture and incubated for 1h. After incubation, the microspheres were washed with PBS buffer (0.01% Tween 20) and dispersed in 400 μL of BSA solution (1%, PBS). 20 μL of FITC-goat anti-mouse IgG was added to the solution and the mixture was incubated for 1h. The microspheres were again washed and diluted with PBS buffer (0.01% Tween 20) for flow cytometric analysis. For BSA control group, no mouse IgG was added, and the test was performed with the same procedures.

Writing-erasing cycles

The MF resin fluorescent shell and conjugated proteins on the microspheres could be removed (erasing) by the same calcination process we used to remove the urea-formaldehyde resin. The microspheres after calcination could be applied to prepare magnetic fluorescent-encoded microspheres again (writing). Magnetic microspheres with 0, 1, 2, and 3 writing-erasing cycles were referred as MS0, MS1, MS2, and MS3, respectively. Accordingly, magnetic fluorescent-encoded microspheres obtained with MS0, MS1, MS2, and MS3 magnetic microspheres were referred as MS0-MF, MS1-MF, MS2-MF, and MS3-MF, respectively.

Results and discussion

Preparation of magnetic fluorescent-encoded MS-MF microspheres

We previously reported the preparation of narrow-dispersed magnetic microspheres via urea-formaldehyde polymerization-induced Fe_3O_4 nanoparticle aggregation.[25] In the present work, we adopted the same method with a slight modification to prepare the magnetic MS microspheres (Fig.1). In contrast to our former work, silica sol was added to reduce the overall light absorption of the magnetic microspheres and enhance the mechanical properties simultaneously. The magnetic microspheres were composed of γ-Fe_2O_3 and silica nanoparticles. The saturation magnetization of the magnetic microspheres could be adjusted from 7.9 emu·g^{-1} to 42.1 emu·g^{-1} by varying the silica sol and ferrofluid ratios as reported.[26] Although certain saturation magnetization decreased with the addition of silica sol, it was sufficient for efficiently magnetic separation of the microspheres. Magnetic nanoparticles (Fe_3O_4 or γ-Fe_2O_3) may influence the fluorescence intensity for their broad light absorption in the visible spectrum and fluorescence quenching through electronic coupling and energy transfer.[30] These problems could be minimized by using near-infrared fluorescence and increasing the distance between the magnetic nanoparticles and fluorescence emitters. For our magnetic fluorescent-encoded microspheres, a silica shell was coated on the magnetic microspheres to separate the magnetic core and fluorescent MF shell, thus eliminating the light quenching of the magnetic core. A further calcination process was conducted to ensure that the MS microspheres could endure high temperature without property changes. Different with most of the magnetic microspheres which are usually polymer microspheres embedded or adsorbed with magnetic nanoparticles,[31] the prepared magnetic MS microspheres comprised of pure inorganic γ-Fe_2O_3 and silica. This trait renders the magnetic MS microspheres with high temperature resistance.[32]

Fluorescent-encoded MF microspheres were prepared in our former work by quantitatively incorporating various dyes at different doping concentrations during the microsphere preparation.[24] The possible dye doping mechanism was investigated and discussed. Dye molecules were initially associated with the branched MF pre-polymer molecules,[33, 34]

and subsequently trapped in the microsphere matrix during further pre-polymer molecule cross-linking and aggregation. Various dyes with different physical and photoluminescence properties could be incorporated into the MF microspheres with encapsulation rates higher than 99%. Microsphere arrays with 25 and 27 sets of encoded emissions were prepared by using two dyes with five different concentrations each and three dyes with three different concentrations each. High encoding capacity could be obtained by adjusting the number and intensity levels of dyes. The fluorescent-encoded MF microspheres showed scarcely dye leakage and good thermal and mechanical stabilities. In the current study, magnetic fluorescent-encoded microspheres were prepared based on our previous works. Fluorescent-encoded MF shell was coated on the magnetic MS microspheres. Rh 110 and SRh 101 were used as representative dyes to verify the MF shell coating. The magnetic fluorescent-encoded MS-MF microspheres of single dye doped with different dye concentrations and two dyes doped with different ratios were obtained.

Magnetic fluorescent-encoded microspheres can be prepared by several methods. Nie and his colleagues have conducted many significant works in fluorescent-encoded microspheres via solvent swelling and hydrophobic interaction.[21, 35, 36] They also utilized hydrophobic interaction to embed quantum dots (QDs) and iron oxide nanocrystals into mesoporous silica beads to prepare bi-functional microspheres.[30] Wilson and colleagues used layer-by-layer (LBL) self-assembly of both water-soluble and hydrophobic CdSe@ZnS QDs on commercial Dynal paramagnetic polystyrene microspheres.[37, 38] Cooper et al. reported the LBL biological self-assembly of streptavidin- and biotin-functionalized QDs on streptavidin-coated Dynal magnetic beads.[39] Chang et al. utilized the solvent swelling and high-temperature swelling for encapsulating magnetic nanoparticles and QDs, respectively, into porous poly(styrene-co-EGDMA-co-MAA) beads.[40] Dou et al. used membrane emulsification-solvent evaporation approach for embedding both QDs and magnetite nanoparticles simultaneously into poly(styrene-co-maleic anhydride) microspheres.[41] Microfluidic devices were also utilized to prepare magnetic fluorescent-encoded microspheres.[42, 43] Most of the magnetic fluorescent-encoded microspheres reported in literature were encoded with QDs. Although QDs have several advantages, such as narrow emission spectra, high photostability, and brightness, fluorescent dyes are much cheaper, easier to handle, and of better batch uniformity. Commercially available magnetic fluorescent-encoded microspheres (Bio-Rad Corp.) were prepared by incorporating two fluorescent dyes at different ratios via solvent swelling and subsequent magnetic nanoparticle coating. [18] The method we used to prepare the magnetic fluorescent-encoded microspheres was simple and direct with relatively cheap materials. The MS-MF microspheres possess a distinct magnetic core/fluorescent shell structure separated by an intermediate silica shell. The most unique characteristic of the MS-MF microspheres was that the inner magnetic core was composed of inorganic γ-Fe_2O_3 and silica, thus could endure high-temperature calcination. This characteristic enables removal of fluorescent-encoded MF shell through calcination while the inner magnetic cores remain unchanged and could be re-coated with new fluorescent-encoded MF shell again.

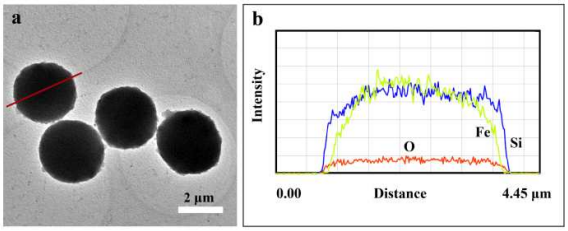


Fig.1 TEM image (a) and EDS line scan curves (b), showing Fe, Si and O element distribution indicated by a line in (a).

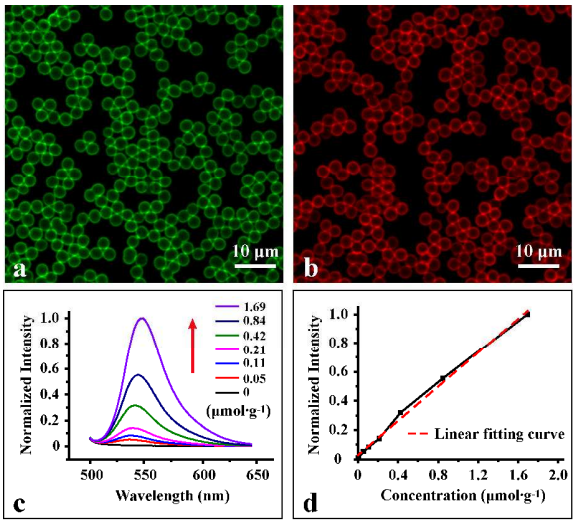


Fig.2 LSCM images of Rh 110 doped (a) and SRh 101 doped (b) magnetic fluorescent microspheres under 488 nm and 555 nm excitation respectively. (c) The normalized emission spectra of Rh 110 doped magnetic fluorescent microspheres with different doping concentrations under 488 nm excitation. (d) The fluorescence emission intensity versus doping concentration curve of Rh 110 doped magnetic fluorescent microspheres (black line) and the linear fitting curve (red dash line).

Characteristics

TEM and energy dispersive spectroscopy (EDS) were used to measure the morphology and component distributions of the microspheres. MS microspheres were of uniform size (Fig.1a). EDS line scan curves were employed to investigate the element distributions (Fig.1b). The Si intensity distribution was wider than the Fe intensity distribution, indicating the outer silica layer and inner magnetic core of the MS microspheres. Fig.2a and 2b show the LSCM images of Rh 110 doped and SRh 101 doped magnetic fluorescent MS-MF microspheres, respectively. The MS-MF microspheres were of uniform size and homogeneously covered with fluorescent MF shell. Each MS-MF microsphere had similar fluorescence intensity. Through adjusting the dye concentrations in pre-polymer solution, magnetic fluorescent microspheres with 6 different fluorescence intensities could by obtained (Fig.2c). The

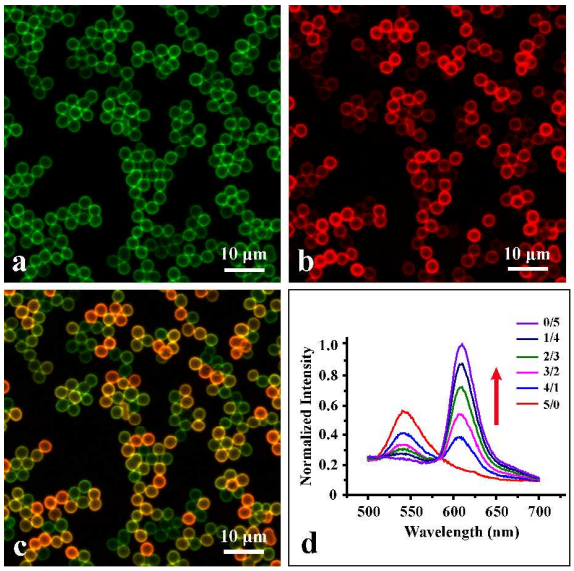


Fig.3 (a-c) LSCM images of magnetic fluorescent-encoded microspheres with six different Rh 110/SRh 101 ratios under 488 nm excitation, (a) Rh 110 channel, (b) SRh 101 channel, (c) merged channel. (d) Corresponding fluorescence emission spectra of the magnetic fluorescent-encoded microspheres under 380 nm excitation.

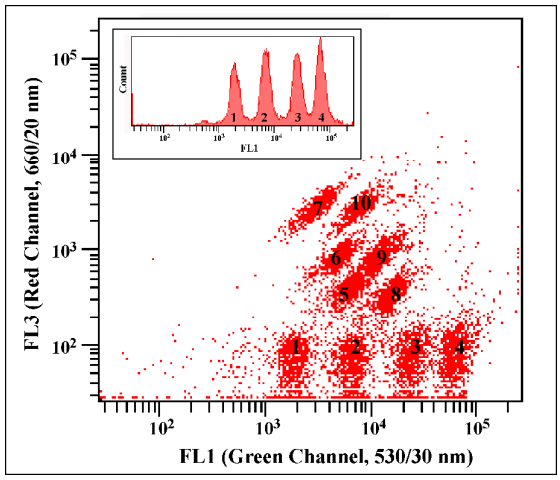


Fig.4 Dot plot for the mixture of ten sets of different encoded microspheres under 488 nm excitation. 1-4: magnetic flurescent-encoded MS-MF microspheres with Rh 110 concentrations of 0.025, 0.10, 0.40, 1.60 μmol·g^{-1}; 5-10: magnetic flurescent-encoded MS-MF microspheres with two Rh 110 concentrations [0.25 μmol·g^{-1} (sets 5, 6, 7), 0.75 μmol·g^{-1} (sets 8, 9, 10)] and three SRh 101 concentrations [0.025 μmol·g^{-1} (sets 5, 8), 0.075 μmol·g^{-1} (sets 6, 9), 0.25 μmol·g^{-1} (sets 7, 10)]. Inset: histogram plot for 1-4 sets of encoded microspheres.

fluorescence emission intensities were linearly proportional to the dye doping concentrations in a wide range, with R^2 = 0.994 (Fig.2d). This result agrees well with our former work and demonstrated the possibility of using different dye concentrations to encode the magnetic microspheres. The LSCM image of the magnetic fluorescent microspheres with different Rh 110 doping concentrations is shown in Fig.S1. These microspheres with different dye doping concentrations could be easily distinguished from one another according to their fluorescent intensities.

As reported in our former study, various dyes with different physical and photoluminescence properties could be simultaneously incorporated into the monodispersed MF microspheres. Multicolor magnetic fluorescent-encoded microspheres were successfully prepared in the present paper by co-doping Rh 110 and SRh 101 with six different ratios in the MF shell (Fig.3a-3c). Six sets of fluorescent-encoded microspheres could be easily distinguished in Fig.3c. The color of the microspheres changed along with different dye ratios. The fluorescence emission intensities were proportional to the dye ratios in the pre-polymer solution (Fig.3d). The optimal excitation wavelengths of Rh 110 and SRh 101 were different. To achieve similar fluorescence emission intensity for both dyes, the excitation wavelength was set to 380 nm. With the increasing proportion of SRh 101 in the pre-polymer solution, its fluorescence emission intensity increased, whereas the emission intensity of Rh 110 decreased correspondingly. These results proved that the encoding strategies used to prepare the fluorescent-encoded MF microspheres are also applicable in the preparation of magnetic fluorescent-encoded microspheres. Fluorescent MF shell was also successfully coated on commercial carboxylic polystyrene microspheres, indicating that this encoding method is also applicable for other systems (Fig.S2).

With the increase of dye concentrations in MF matrix, the average inter-distance of doped dyes decreases accordingly. The resonance energy transfer (RET) between the doped dyes becomes strong and could be exploited to achieve the fluorescence excitations of multiple dyes under the optimum excitation wavelength of primary donor dye. Fig.4 shows the flow cytometric analysis results for ten sets of encoded microspheres: four sets of microspheres doped with different Rh 110 concentrations and six sets of microspheres doped with combinations of two Rh 110 concentrations and three SRh 101 concentrations. Encoded microspheres were spatially separated into ten clusters according to their fluorescence intensities, which demonstrated their coding capacity for multiplex detection. For sets of 5-10, the RET effects could be easily observed. The fluorescence intensity of set 5 (Rh 110 doping concentration of 0.25 μmol·g^{-1}) was similar to set 2 (Rh 110 doping concentration of 0.10 μmol·g^{-1}), which indicated that the co-doping with SRh 101 significantly decreased the fluorescence intensity of Rh 110. With the increasing of SRh 101 concentration, the RET became more significant, accordingly the fluorescence intensities of Rh 110 further decreased. This led to the non-vertical alignments of sets of 5, 6 and 7. This situation was also similar to the sets of 8, 9 and 10.

The FTIR spectra of MS, MF, and MS-MF microspheres are shown in Fig.5a. Compared with the magnetic microspheres in our previous paper, the specific absorption band of Si-O-Si asymmetric stretching vibration at 1087 cm^{-1} was significantly enhanced for MS and MS-MF microspheres.[25] The enhanced absorption band was attributed to the added silica sol and further silica shell coating. The specific absorption band of

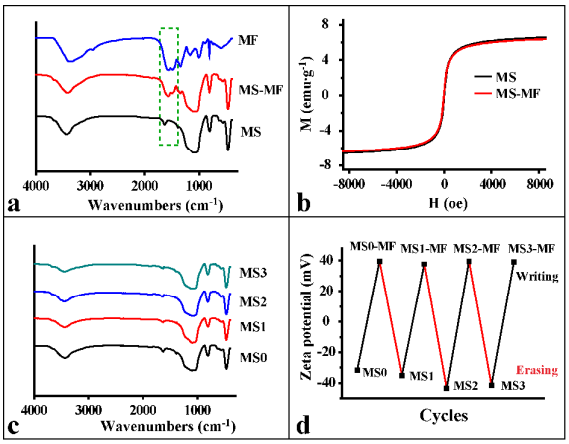


Fig.5 (a) FTIR spectra of MS, MF and MS-MF microspheres. (b) The magnetic hysteresis loops of MS and MS-MF microspheres. (c) FTIR spectra of magnetic MS microspheres with different writing-erasing cycles. (d) Reversible zeta potential changes of microspheres upon the writing-erasing cycles.

1,3,5-striazine ring at 1556 cm^{-1} demonstrated the successful coating of the MF shell on MS microspheres.[44] The saturation magnetization of MS-MF microspheres was 6.40 $emu \cdot g^{-1}$, which was slightly lower than that of the MS microspheres. The saturation magnetization was similar to the values reported in literature,[5, 41] endowing the MS-MF microspheres with strong magnetic responsiveness. Under external magnetic field, the originally dispersed MS-MF microspheres orderly aligned along the magnetic field line (Fig.6b). The MS-MF microspheres dispersed in the aqueous solution could be rapidly separated by a handheld magnet (Fig.6d). These results demonstrate that the MS-MF microspheres could be manipulated by the external magnetic field.

Recycling of MS-MF microspheres and writing-erasing cycles

As described above, the recycling and regeneration of the magnetic fluorescent-encoded microspheres remain difficult. In this study, we used a simple method—calcination to remove the encoding and conjugated biomolecules simultaneously, while maintaining the inner MS microspheres unaffected which could be re-encoded again. The writing-erasing cycles were repeated three times to verify that the MS microspheres could be repeatedly recycled. More repetitions are also possible if necessary. The writing-erasing cycles were monitored using the FTIR spectra analysis. The specific absorption band of 1,3,5-striazine ring at 1556 cm^{-1} (MS0-MF, Fig.S3) disappeared after calcination (MS1, Fig.5c), which proved the successful removal of the MF shell. The MS1 microspheres were subjected to MF shell coating again. The specific absorption band of 1,3,5-striazine ring at 1556 cm^{-1} confirmed the MF shell re-coating (MS1-MF, Fig.S3). The MS microspheres with different cycles were of similar FTIR spectra, and all could be subjected to MF shell coating again. The surface zeta potentials during writing-erasing cycles were also monitored (Fig.5d). The negative zeta potential of MS microspheres from −31.7 mV changed to positive after MF coating, which also demonstrated the successful MF coating.

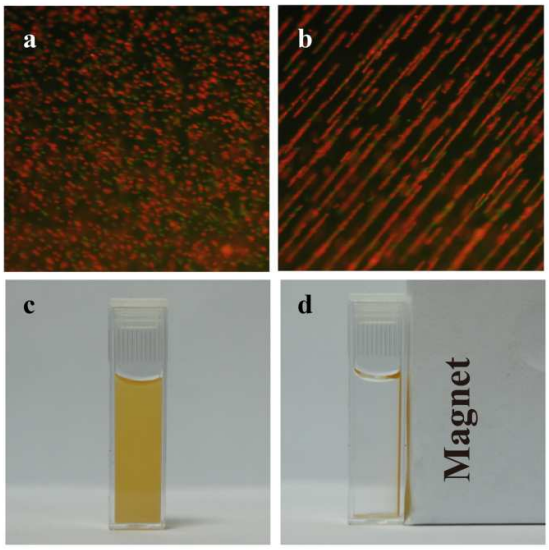


Fig.6 (a) The fluorescence microscope images of multicolor magnetic fluorescent-encoded microspheres and (b) their ordered alignment in the presence of an external magnetic field. (c) The photographs of MS-MF microspheres and (d) their magnetic responsiveness.

After calcination, the zeta potential changed to negative (−35.0 mV) again, demonstrating the removal of the MF shell. The zeta potential reversibly changed upon the writing-erasing cycles. MS microspheres with different writing-erasing cycles showed similar negative zeta potentials, and the MS-MF microspheres with different cycles showed similar positive zeta potentials (39.0 mV). These results confirmed that the MS microspheres remained unaffected after writing-erasing cycles and could be repeatedly applied to the MF shell coating.

SEM was employed to examine the morphologies of MS and MS-MF microspheres with different writing-erasing cycles (Fig.7). The MS microspheres with different cycles were all spherical with no obvious particle size changes or morphology damages. The MS-MF microspheres with different cycles were uniform with smooth surfaces. The diameters of MS-MF microspheres with 0, 1, 2, and 3 cycles were 2.88±0.18, 2.91±0.19, 2.89±0.18, and 2.90±0.19 μm, respectively. No agglomeration was observed, which demonstrated the uniform MF coating on the MS microspheres. The magnetic fluorescent-encoded MS-MF microspheres were also

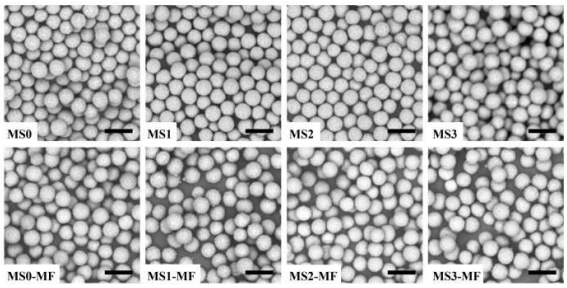


Fig.7 SEM images of magnetic MS microspheres and magnetic fluorescent-encoded MS-MF microspheres with different writing-erasing cycles. Scale bar 5 μm.

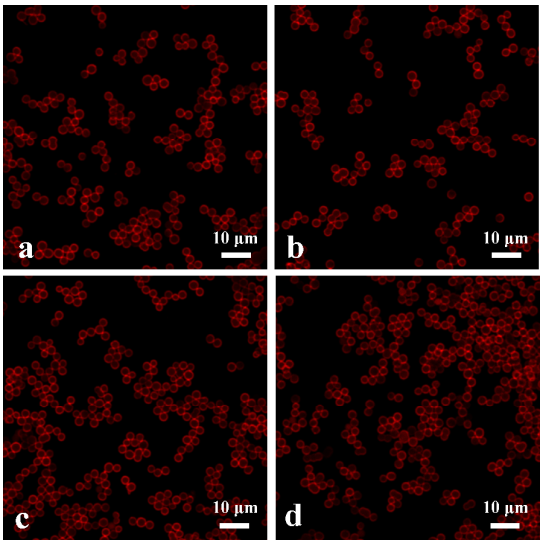


Fig.8 LSCM images of magnetic fluorescent-microspheres with different writing-erasing cycles. (a) MS0-MF, (b) MS1-MF, (c) MS2-MF, (d) MS3-MF.

examined by the LSCM (Fig.8). All MS-MF microspheres with different cycles showed similar and uniform fluorescence intensities. The fluorescence intensities were quantitatively measured using a fluorescence spectrophotometer (Table 1). The fluorescence intensities of Rh 110 doped and intensity ratios of SRh 101/Rh 110 dual-doped MS-MF microspheres after writing-erasing cycles were consistent with those of MS0-MF microspheres. The detailed spectrum information is provided in Fig.S4 and S5. The fluorescence emission spectra of SRh 101 doped and SRh 101/Rh 110 dual-doped MS-MF microspheres with different writing-erasing cycles were similar. These results demonstrated that the MS microspheres after writing-erasing cycles were rewritable. The magnetic properties of MS-MF microspheres were also measured (Fig.S6), and the results showed that the MS-MF microspheres were all superparamagnetic and had similar saturation magnetizations.

Table 1 Characterizations of magnetic fluorescent-encoded MS-MF microspheres with different writing-erasing cycles

	MS0-MF	MS1-MF	MS2-MF	MS3-MF
Emission intensity of SRh 101 (10^6, a.u.)	2.88±0.12	2.91±0.09	2.85±0.08	2.80±0.13
Emission intensity ratio of SRh 101/Rh 110	3.06±0.01	3.16±0.01	3.14±0.02	3.13±0.01
BSA conjugation (mg protein per g microspheres)	5.83±0.10	5.07±0.27	5.39±0.96	5.36±0.65

The successful recycling of magnetic fluorescent-encoded microspheres in this study was attributed to several reasons. First, the prepared magnetic fluorescent-encoded MS-MF microspheres were of distinct core/shell structure. The inner inorganic magnetic core could endure high-temperature calcination without property changes. Second, the MF shell, organic dyes, and bioconjugation reagents were all organic molecules and could be easily removed via calcination. Third, the magnetic MS microspheres after writing-erasing cycles could be applied to the fluorescent MF shell coating repeatedly. These characteristics combined with the simple and low-cost preparation process, ensured the recycling and regeneration of the magnetic fluorescent-encoded microspheres. Those magnetic fluorescent-encoded microspheres in previous studies cannot be recycled via calcination because most of them were polymer microspheres or encoded with inorganic QDs. The magnetic field assisted collection and calcination method was rather simple and low-cost. As almost no reagent costs are involved in recycling and regeneration of magnetic microspheres, over one-third of the costs for magnetic microspheres could be saved. All the molecules (including capture probes, targets and reporter molecules) and encoding information could be removed simultaneously and completely. The recycled MS microspheres could also be used in other areas after surface modification. Polydopamine has been widely used for its versatility for various substrates and convenience for functionalization[45]. Biomolecules and noble metal nanoparticles could be readily immobilized on the polydopamine surfaces[46, 47]. In the present paper, the recycled MS microspheres were successfully modified with polydopamine coating (Fig.S7). After surface functionalization, the MS microspheres could be used as magnetic carriers in various applications. The recycling and regeneration of magnetic microspheres will greatly decrease the reagent costs and avoid polluting the environment simultaneously.

Protein conjugation and sandwich immunoassay

Receptor molecules (e.g., antibodies and nucleic acids) must be conjugated to the surfaces of the MS-MF microspheres to satisfy the requirements for multiplex analyses. BSA molecules were firstly adsorbed on positive MS-MF microspheres through electrostatic interaction. The adsorbed BSA molecules were cross-linked and modified with GA. GA is widely used in bioconjugation for its effectiveness and gentle reaction conditions, which showed slight effects on the biomolecular activities.[28, 29] GA reacted with the amino groups of BSA and endowed the microspheres with reactive aldehyde groups, which could readily react with the amine groups of numerous molecules. BSA was conjugated on the GA-modified MS-MF microspheres to verify the efficiency of the electrostatic adsorption and covalent attachment method. The amounts of cross-linked BSA on microspheres were determined with standard protein quantitative kit (Table 1). The MS-MF microspheres with different cycles showed similar protein conjugation. The average surface coverage of BSA on MS-MF microspheres was about 5.75 $mg \cdot m^{-2}$, assuming the density of the MS-MF microspheres was equal to silica. The surface coverage of protein was similarly with other literatures, for example 1.6 $mg \cdot m^{-2}$ of anti-IgG on quantum dot-labeled polystyrene beads.[48, 49] Goat anti-mouse IgG was also conjugated on the MS-MF microspheres. The MS-MF microspheres after immunoassay were analyzed via flow cytometry (Fig.9). The encoding information of MS-MF

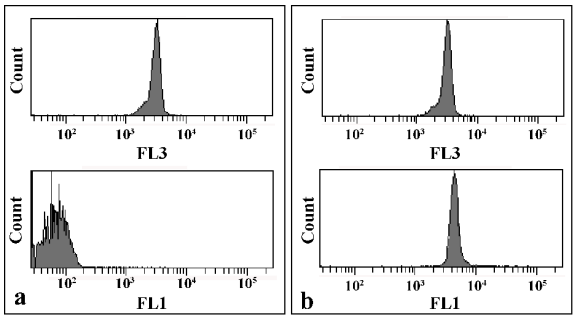


Fig.9 Flow cytometry analysis for SRh 101 doped magnetic fluorescent-encoded MS-MF microsphere-based sandwich immunoassay. a: the fluorescent intensities for microspheres of BSA control group; b: the fluorescent intensities for microspheres of IgG group.

microspheres were measured on FL3 channel and the fluorescent intensities remained constant after immunoassay. The FITC fluorescent intensities were measured on FL1 channel. The fluorescent intensity of microspheres of IgG group significantly increased than BSA group, which confirmed the higher specific antigen-antibody reaction on microspheres. These results demonstrated that the electrostatic adsorption and GA covalent attachment method was effective for surface conjugation, and the magnetic fluorescent-encoded MS-MF microspheres had the potential to be applied in multiplex analyses. Compared with other surface modification methods, such as silane coupling and dopamine polymerization, this electrostatic adsorption and covalent attachment method was straightforward without affecting the fluorescence intensities. All the reagents used in this method were organic, which ensured the complete removal via calcination.

Conclusions

We developed a novel method to prepare rewritable magnetic fluorescent-encoded MS-MF microspheres based on our previous works. The MS-MF microspheres were of unique magnetic core/fluorescent shell structure with an intermediate silica layer, and high encoding capacity could be achieved by varying the dye types and concentrations. The preparation and recycling process of the MS-MF microspheres were simple and low-cost. The fluorescent MF shell and bound biomolecules could be simultaneously removed via calcination in air. The rewritable MS-MF microspheres could be re-encoded with different fluorescence information and utilized multiple times, thereby significantly decreasing the analysis cost. The recycled MS microspheres could be used not only in rewritable magnetic fluorescent-encoded microspheres but also in other areas as fluorescent or magnetic carriers after appropriate modification.

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Rewritable Magnetic Fluorescent-Encoded Microspheres: Preparation, Characterization, and Recycling

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Rewritable magnetic fluorescent-encoded microspheres were prepared by coating magnetic microspheres with fluorescent-encoded shell which could be erased by calcination.