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Multistage Magnetic Separation of Microspheres Enabled by Temperature-Responsive Polymers

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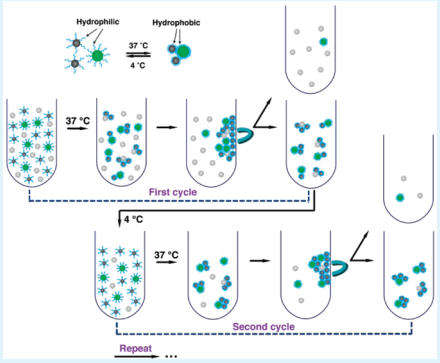
ABSTRACT: A simple and rapid method for separation of cells is to functionalize magnetic particles with a receptor that selectively captures the target and then pull the magnetic particles out of the mixture upon applying a magnetic field. The separation efficiency of magnetic separation, however, is typically limited by the nonspecific interaction between the magnetic particles and nontarget species. We here present a multistage separation process that is able to effectively circumvent the problem caused by the nonspecific interactions by introducing multiple capture-and-release cycles to the magnetic separation process. The multiple capture-and-release cycles are enabled by attaching a temperature-responsive polymer to both the magnetic particles and the targets. Through temperature cycling, we demonstrate that target microspheres can be separated from nontarget microspheres in multiple separation stages. The overall enrichment factor significantly increases with the number of separation stages and reaches as high as 1.87×10^5 after 5 cycles.



KEYWORDS: *magnetic separation, multistage separation, thermal-responsive polymer, poly(N-isopropylacrylamide), nonspecific interaction, magnetic nanoparticles*

INTRODUCTION

Magnetic separation provides a rapid and simple method for efficient and reliable separation of cells.[1−6] It utilizes magnetic particles to attach to specific cells and separate them from a heterogeneous mixture upon applying an external magnetic field. Typically, the magnetic particles are functionalized with a receptor, which captures the cells selectively, and high separation efficiency is enabled by the extremely high binding affinity and specificity of the receptor, such as an antibody, immobilized on the magnetic particles. However, a major obstacle that prevents the magnetic separation technology from achieving adequate separation efficiencies is nonspecific interactions between the cells and magnetic particles. For example, in magnetic cell separation processes that require very large enrichment factors, although the binding affinity of antibodies with the antigens is orders of magnitude greater than the nonspecific binding, the effect of nonspecific interactions becomes significant and eventually becomes a major challenge.[7−9]

To circumvent the challenge caused by nonspecific interactions in current single-stage magnetic separation techniques, we here present a multistage separation scheme that is able to yield high enrichment factors by introducing multiple capture-and-release cycles to the magnetic separation process. Poly(N-isopropylacrylamide) (PNIPAM) is the key molecule employed in this work to enable the reversible capture-and-release cycles using magnetic particles. PNIPAM is a stimuli-responsive polymer that exhibits a well-known temperature-responsive phase transition in aqueous solution at its lower critical solution temperature (LCST) of about 32 °C.[10−12] It is hydrophilic assuming a random coil conformation when the temperature is below its LCST in water, but becomes hydrophobic and aggregated with collapsed globule conformation in aqueous solutions when the temperature is above LCST.[10−12] This reversible temperature-responsive phase transition has been utilized for the separation and purification of cells,[13−15] proteins,[16−18] nucleic acids,[19,20] and other biomolecules.[21] In the present study, the multiple capture-and-release cycles are enabled by attaching PNIPAM to both the magnetic nanoparticles (MNPs) and the targets and manipulating the reversible hydrophobic interactions between such functionalized MNPs and targets. Through temperature cycling, which triggers the reversible hydrophobic-to-hydrophilic phase transition of PNIPAM, we demonstrate that PNIPAM-functionalized polystyrene (PS) microspheres can be separated from bare (nonfunctionalized) PS microspheres in multiple separation stages, and the enrichment factor significantly increases with the number of separation stages. The result indicates that the multistage separation scheme can effectively circumvent problems caused by nonspecific interactions and significantly improve separation efficiencies of magnetic separation technologies.

DESIGN OF MULTISTAGE SEPARATION PROCESS

Figure 1 schematically shows the multistage separation process. As a proof of demonstration, PNIPAM-functionalized fluo-

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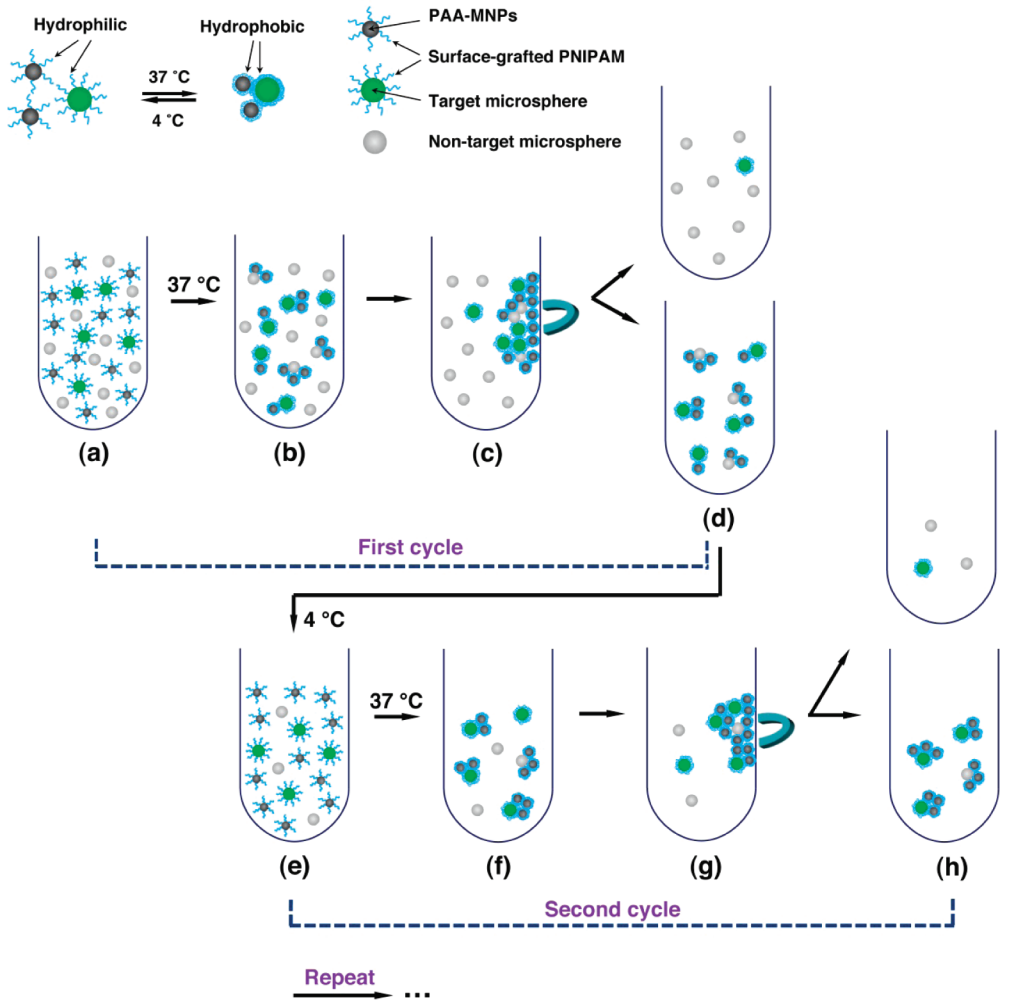

Figure 1. Schematic of the multistage magnetic separation process using PNIPAM-functionalized magnetic nanoparticles (PNIPAM-MNPs). (a) PNIPAM-MNPs, target microspheres, and nontarget microspheres are mixed in a 4 °C buffer. (b) Capture of the targets by PNIPAM-MNPs through hydrophobic interactions upon raising the temperature to 37 °C. (c) The MNPs are collected by a magnet. (d) The original mixture is separated into two parts: the pellet contains MNPs, the captured targets, and the nontargets captured due to nonspecific interactions; the supernatant contains the rest of the mixture and is decanted. (e) Release of the targets from PNIPAM-MNPs in a 4 °C buffer. (f–h) Repeat the process of b–d.

rescent PS microspheres are used as the targets and bare carboxylated PS microspheres (with no PNIPAM) are used as the nontargets. It should be noted that in practical separation processes, the PNIPAM may be conjugated to a bioreceptor such as an antibody and thus attached to the target through antibody–antigen interactions. To capture the PNIPAM-functionalized target PS microspheres by using MNPs, we also grafted the surface of the MNPs with PNIPAM. Surface functionalization of MNPs with PNIPAM enables us to reversibly regulate the hydrophobicity of the particle surface by simply cycling the temperature below and above the LCST.

The separation process starts with adding PNIPAM-functionalized MNPs to a mixture of both PNIPAM-functionalized PS microspheres and bare PS microspheres (Figure 1a). Upon raising the temperature to 37 °C (>LCST), the PNIPAM molecules on both the MNPs and the target microspheres undergo a hydrophilic-to-hydrophobic phase transition, and the targets are captured by the MNPs due to the hydrophobic interaction between PNIPAM molecules (Figure 1b). During this process, some of the nontarget bare PS microspheres may also be attached to the MNPs due to nonspecific interactions. Next, the MNPs are magnetically collected in the form of a pellet, which contains the targets captured by the MNPs as well as the nontargets either attached to the MNPs due to nonspecific interactions or embedded into the pellet during the agglomeration of the MNPs (Figure 1c). The pellet is then separated from the supernatant and resuspended in a buffer (Figure 1d).

After this first separation cycle, a mixture of target and nontarget microspheres has been separated into two parts: one with a higher percentage of nontarget microspheres and the other with a higher percentage of target microspheres than those in the original mixture. The percentage increase of the target microspheres in the second mixture may be characterized by using an enrichment factor, which is defined as the increase (typically in the number of folds) in the ratio of target to nontarget microspheres as a result of the separation process. The enrichment factor obtained after one separation cycle is limited by the nonspecific interactions between the PNIPAM molecules and the nontargets. The reversible hydrophobic-to-hydrophilic conformational transition of PNIPAM enables us to improve this enrichment factor by repeating this separation

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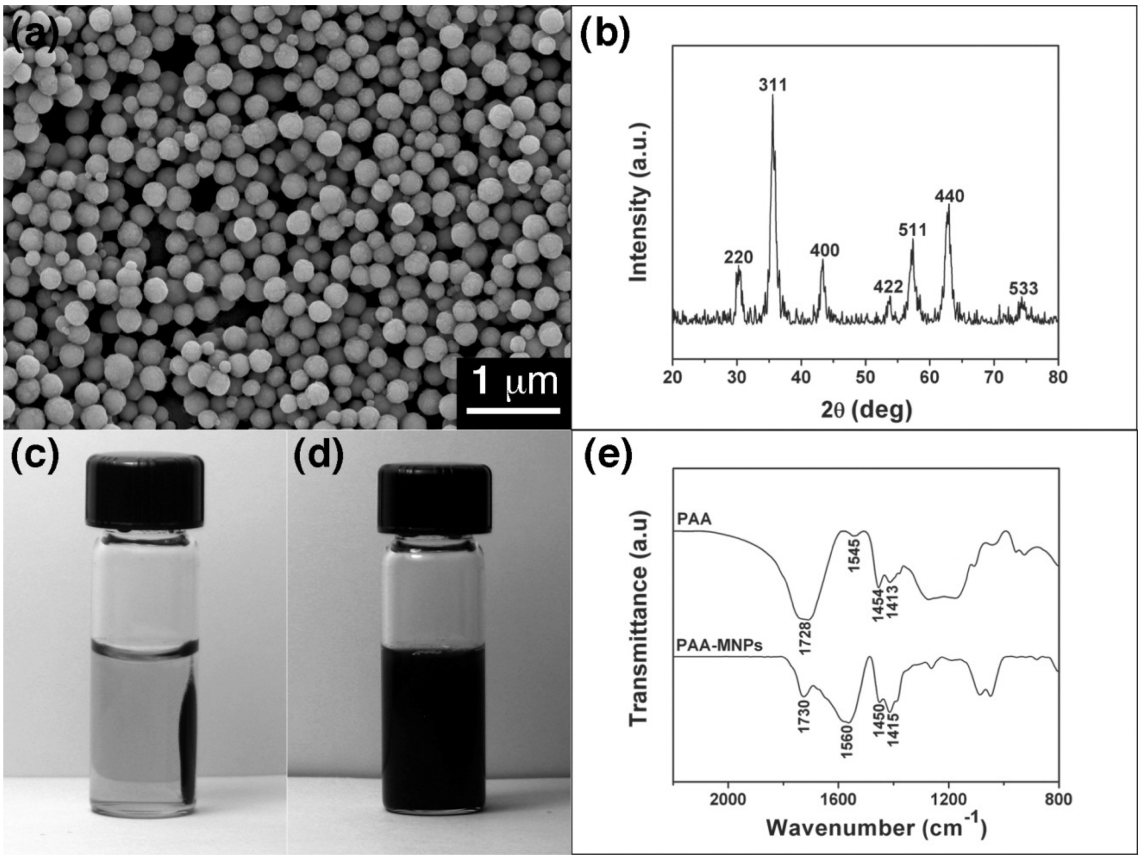

Figure 2. Characterizations of poly (acrylic acid)-modified magnetic nanoparticles (PAA-MNPs). (a) SEM image. (b) XRD pattern. (c) Magnetic property of the PAA-MNPs; the MNPs are attracted to the wall of the vial when a magnet is present. (d) After removing the magnet, the PAA-MNPs are easily redispersed in water with gentle shaking. (e) FTIR spectra of PAA and PAA-MNPs.

process through cycling the temperature. Upon cooling the buffer to 4 °C and redispersion, the PNIPAM on both the MNPs and the target PS microspheres is triggered to its hydrophilic conformation, which detaches the target PS microspheres from the MNPs (Figure 1e) and initiates another separation cycle. Repeating this capture-and-release procedure (Figures 1e–h) multiple times can effectively circumvent problems caused by the nonspecific interactions and significantly improve the separation efficiency. It should be noted that for the purpose of demonstration, our interest in this experiment is to increase the percentage of the target microspheres in the target-rich mixture as we repeat the separation cycle, but the same mechanism is also applicable to increasing the percentage of the nontarget microspheres.

MATERIALS AND METHODS

Materials. Nonfluorescent 4.95 μm carboxylated polystyrene microspheres and 5.78 μm green fluorescent carboxylated polystyrene (PS) microspheres with an excitation wavelength of 480 nm and an emission wavelength of 520 nm were obtained from Bangs Laboratories, Inc. The surface density of carboxyl groups on the fluorescent PS microspheres is about 1.075×10^{18} COOH/m^2. Ethylene glycol was purchased from J.T. Baker. Amino terminated poly (N-isopropylacrylamide) (PNIPAM) (M_n = 45 600 g/mol, PDI = 1.62) were purchased from Polymer Source, Inc. and used as received. Iron(III) chloride ($FeCl_3$), ethanolamine, sodium acetate (NaAc), poly(acrylic acid) (PAA, $M_w \approx$ 2000 g/mol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 2-(N-morpholino)ethanesulfonic acid (MES), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. All buffers were prepared or diluted in deionized water.

Synthesis of Poly(acrylic acid)-Modified Fe_3O_4 Magnetic Nanoparticles (PAA-MNPs). The PAA-MNPs were synthesized by using a solvothermal method. Briefly, $FeCl_3$ (0.8 g) was dissolved in ethylene glycol (40 mL) with vigorous stirring, followed by addition of NaAc (3.6 g) and PAA (1.0 g). The mixture was stirred continuously for 30 min, sealed in a Teflon-lined stainless-steel autoclave, and then reacted at 200 °C for 10 h. After the reaction was finished, the autoclave was cooled to room temperature. The products were collected, washed several times with ethanol, and then dried under a vacuum at 60 °C before characterization and usage.

Characterization of PAA-MNPs. The size and morphology of the as-synthesized PAA-MNPs were characterized using scanning electron microscopy (SEM, Philips XL-30 field, 15 kV). The crystal structures of the PAA-MNPs were examined by using powder X-ray diffraction (XRD) (Philips X'pert Diffractometer using CuKα radiation, λ=1.54178 Å). The chemical composition of the PAA-MNPs was examined by Fourier transform infrared (FT-IR) spectrometry. The sample of PAA-MNPs was washed five times with ethanol, redispersed in water and dried in a powder form. FTIR samples were prepared using a KBr-pellet method, and the spectra were collected by a Bruker Vertex-70LS FT-IR spectrometer.

Conjugation of PAA-MNPs and Green Fluorescent Carboxylated PS Microspheres with PNIPAM. The carboxyl group on the surfaces of PAA-MNPs and green fluorescent PS microspheres was used to covalently link amino-terminated PNIPAM to the surfaces by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) chemistry. The PAA-MNPs (0.5 mL, 2.5%) were washed three times in 0.1 M carbonate buffer (pH 9.6) and then another three times in 0.1 M MES buffer (pH 6.5). After washing, the MNPs were redispersed in MES buffer. To activate the carboxyl groups, fresh solution of EDC (2%, w/v) in MES buffer was added and the mixture was incubated for 3 h at room temperature in the dark. After incubation, the MNPs were washed three times with MES buffer to

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remove the unreacted EDC. Then, the MNPs were redispersed in 0.1 M borate buffer (pH 8.5) and enough amount of amino terminated PNIPAM was added for the functionalization. The mixture was incubated overnight at room temperature in the dark. Following conjugation, the MNPs were magnetically separated and thoroughly washed with PBS (pH 7.4) to remove unbound amino-terminated PNIPAM, and then incubated in the blocking solution (0.3 M ethanolamine in borate buffer) for 30 min to block the unreacted carboxylate sites. The MNPs were then rinsed thoroughly, and finally stored in storage buffer (PBS, 0.1% BSA w/v) with a concentration of 1 mg/mL at 4 °C. In our experiments, the PNIPAM-modified MNPs retained their temperature-responsive property with no noticeable loss of functionality after at least 10 cycles. Similar procedure was used for grafting of amino-terminated PNIPAM to green fluorescent carboxylated PS microspheres.

Reversible Capture-and-Release of Target Microspheres Using PNIPAM-MNPs. Specified amount of PNIPAM-MNPs was added into a sample of PNIPAM-functionalized green fluorescent microspheres suspended in 1 mL 4 °C PBS buffer. The mixture was warmed up to 37 °C and then incubated for 10 min in the dark. The aggregates were then collected by a neodymium−iron-boron magnet. The magnetically collected microspheres were separated from the supernatant and then redispersed in a 4 °C PBS buffer. As a comparison, bare PS microspheres were used in the capture-and-release cycles in the same manner.

Multistage Separation of Microspheres through Reversible Capture-and-Release Cycles. PNIPAM-functionalized green fluorescent microspheres were used as target microspheres, whereas carboxylated bare nonfluorescent polystyrene microspheres were used as nontarget microspheres. Mixtures of target and nontarget microspheres at four different ratios of $1:1 \times 10^2$, $1:1 \times 10^3$, $1:1 \times 10^4$, and $1:1 \times 10^5$, respectively, were used in the experiments. The number of target microspheres was kept at 5×10^6 and the separation was performed in 1 mL buffer. The separation process started with adding a certain amount of PNIPAM-MNPs to the mixture containing both the target and the nontarget microspheres. The mixture was then incubated at 37 °C for 10 min in the dark. After incubation, a magnet was used to pull the MNPs and PS microspheres attached to the MNPs out of the mixture. The magnetically collected particles were then separated from the supernatant, resuspended in the PBS, and incubated for 10 min at 4 °C in the dark. The above capture-and-release cycle was repeated for a specified number of times.

Counting of PS Microspheres. PS microspheres were counted and the numbers of bare (nonfluorescent) and fluorescent PS microspheres in PBS buffer after each capture-and-release cycle were determined using a BD FACSAria flow cytometer (BD BioSciences, San Jose, CA).

RESULTS AND DISCUSSION

The PAA-MNPs were synthesized by a one-step solvothermal method, where PAA in the reaction system acted both as a ligand and a surface functionalization agent. Figure 2a presents an SEM image of the resulting products. It can be seen that the as-synthesized PAA-MNPs are spheres of about 300 nm in diameter with a narrow size distribution. Figure 2b presents the XRD pattern of the product, which can be indexed to Fe_3O_4 (JCPDS 75−1609). The PAA-MNPs were easily dispersed in water by sonication and the dispersion remained stable for more than 0.5 h before precipitation occurred. Upon placement of a magnet next to the vial, the PAA-MNPs were quickly attracted to the magnet and agglomerated at the vial wall within a few seconds, leaving the supernatant transparent (Figure 2c). After removing the magnet, the PAA-MNPs were easily redispersed in water with gentle shaking (Figure 2d). Figure 2e compares the FT-IR spectra of pure PAA and PAA-MNPs. Both spectra show bands at 1730, 1450, 1413, and 1560 cm^{-1}, which are characteristics of the PAA—the band at 1730 cm^{-1} is characteristic of the C═O stretching mode for the protonated carboxylate group, and the other 3 bands are associated with the CH_2 bending mode, symmetric and asymmetric C−O stretching modes of the COO^- group, respectively.[22−24] These results indicate the presence of PAA on the surface of the MNPs even after extensive washing. The carboxyl groups on the surface of PAA-MNPs were used to covalently attach amino-terminated PNIPAM to the MNPs, which were employed to separate PNIPAM-functionalized microspheres from bare microspheres in the subsequent experiments.

We first examined the effect of the concentration of PNIPAM-MNPs on the capture efficiency of PNIPAM-functionalized target microspheres. In this set of experiments, PNIPAM-functionalized target microspheres were mixed with PNIPAM-MNPs in different concentrations. The final concentration of PNIPAM-functionalized microspheres in all samples was 5.0×10^6 microspheres/mL and the concentration of PNIPAM-MNPs was varied from 0.005 to 0.12 mg/mL. A capture-and-release cycle was carried out as described in the experimental design. The steps were the same as those shown in Figure 1a−e except that the mixture did not contain nontarget microspheres in this set of experiments. The capture efficiency was determined by measuring the percentage of the target microspheres left in the mixture after the cycle. Figure 3 plots the capture efficiency as a function of the concentration of PNIPAM-MNPs employed at the start of the process. It is observed that the capture efficiency increases from ∼22% to ∼92% when the concentration of the PNIPAM-MNPs is increased from 0.005 to 0.03 mg/mL, after which a plateau occurs——further increase of the PNIPAM-MNP concentration does not significantly affect the capture efficiency of target microspheres. This indicates that for a certain concentration of target microspheres, the concentration of MNPs is not critical as long as a high enough concentration of MNPs is used. For multiple capture-and-release cycles, this implies that if a high enough concentration of MNPs is used for the first separation cycle, the concentration of MNPs does not need to be adjusted for subsequent separation cycles as the total number of target microspheres slightly decreases due to the less than unity capture efficiencies. For the subsequent experiments conducted in this work, the concentration of target microspheres was kept at 5.0×10^6 microspheres/mL or less

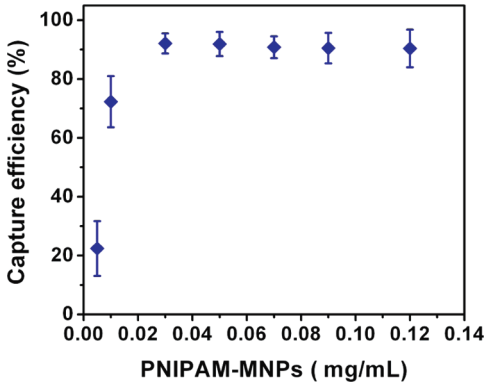

Figure 3. Effect of the concentration of PNIPAM-MNPs on the capture efficiency of the target microspheres. The initial concentration of target microspheres is 5.0×10^6 microspheres/mL. For each sample, one capture-and-release cycle is performed. Data are presented as the mean and standard deviation from five independent experiments.

and, therefore, 0.05 mg/mL PNIPAM-MNPs was used in the separation processes.

We next examined the selectivity of one capture-and-release cycle by comparing the number of PNIPAM-functionalized target microspheres with that of the nontarget carboxyl-terminated microspheres captured and released in the process. For such comparison, we started with two mixtures made by adding the same amount of PNIPAM-MNPs to suspensions of target microspheres and nontarget microspheres, respectively. In both mixtures, the final concentration of the microspheres was about 5.0×10^6 microspheres/mL and the concentration of the PNIPAM-MNPs was 0.05 mg/mL. After raising the temperature and magnetically collecting the MNPs (as schematically shown in Figure 1a–d), the PS microspheres in the supernatant (Figure 1d) of each mixture were counted and the percentage of PS microspheres captured by the MNPs in the pellet was calculated. Afterward, the pellet was redispersed in 4 °C buffer, and the PS microspheres released from the MNP in the buffer were counted. Figure 4 presents the

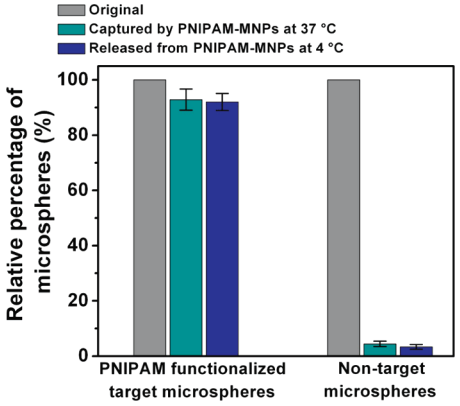

Figure 4. Comparative study on the capture-and-release specificity of PNIPAM-functionalized target microspheres and nontarget microspheres (bare PS microspheres) using PNIPAM-MNPs. The initial concentrations of target and nontarget microspheres are 5.0×10^6 and 4.9×10^6 microspheres/mL, respectively. The percentages of microspheres counted after each capture or release process are presented using the number of microspheres in the original sample as a reference. The average and standard deviation are calculated from five independent data sets.

percentages of the target PS microspheres captured and released by the MNP in comparison to those of the nontarget PS microspheres. The data are based on five independent experiments. In average, about 92% of the target microspheres were captured by the MNPs, and about 91% of the target microspheres were released after the capture in one capture-and-release cycle. In comparison, only less than 5% of the nontarget microspheres were left in the mixture after one capture-and-release cycle.

The large difference between the percentages of target and nontarget PS microspheres left in the mixture after one capture-and-release cycle and the capability of reversibly tuning the hydrophobicity of the PNIPAM molecule on both the target PS microspheres and the MNPs enable us to develop a multistage separation technique by repeating the capture-and-release cycles. To demonstrate the capability of such a technique, we mixed target microspheres with nontarget microspheres at various ratios of $1{:}1 \times 10^2$, $1{:}1 \times 10^3$, $1{:}1 \times 10^4$, and $1{:}1 \times 10^5$, and a specified number of separation cycles were carried out with each sample. The overall enrichment factors $k_{overall}$ (defined as the increase, in the number of folds, of the ratio of target to nontarget microspheres as a result of the separation cycles) obtained with each sample after up to 5 capture-and-release cycles are presented in Figure 5. It is evident that the

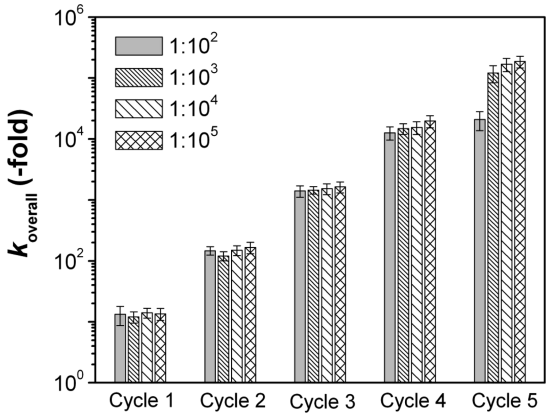

Figure 5. Overall enrichment factor $k_{overall}$ as a function of the separation cycles for mixtures with various initial ratios of target to nontarget microspheres. The mean and standard deviation are calculated from five independent data sets.

overall enrichment factors significantly increase with the number of capture-and-release cycles. For example, one capture-and-release cycle was able to increase the ratio of the target to nontarget microspheres ($R_{T/NT}$) from initially $1{:}1 \times 10^2$ (or 0.01) to 0.133; 3 capture-and-release cycles were able to increase it to 14.05; and 5 cycles were able to increase it to 2100. Correspondingly, the enrichment factors were 13.3, 1,405, and 2.10×10^4 after one, three, and five capture-and-release cycles, respectively. The significance of multiple separation cycles becomes more apparent when the initial $R_{T/NT}$ decreases. For example, overall enrichment factors of 1.21×10^5, 1.69×10^5, and 1.87×10^5 were obtained after 5 capture-and-release cycles when the initial $R_{T/NT}$ was $1{:}1 \times 10^3$, $1{:}1 \times 10^4$, and $1{:}1 \times 10^5$, respectively. Figure 6 plots the single cycle enrichment factor $k_{single\text{-}cycle}$ versus $R_{T/NT}$ for each cycle based on the data obtained from all four samples. The $k_{single\text{-}cycle}$ appears to decrease as $R_{T/NT}$ increases, which implies that further enriching the target microspheres becomes more difficult as their concentration increases. Therefore, smaller

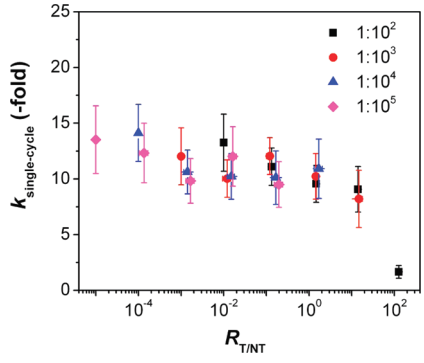

Figure 6. Single-cycle enrichment factor $k_{single\text{-}cycle}$ versus the ratio of target to nontarget microspheres ($R_{T/NT}$) before each cycle calculated for samples with 4 different initial $R_{T/NT}$ values. The mean and standard deviation are calculated from five independent data sets.

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overall enrichment factors are expected as the initial $R_{T/NT}$ increases.

The above experiments demonstrate the feasibility of the multistage separation scheme. For demonstration purposes, the temperature-responsive molecule used in our experiment is PNIPAM and it is covalently attached to the target PS microspheres. In practical applications, selective attaching of PNIPAM to the targets may be realized by linking the PNIPAM molecule to an antibody,[25] which selectively captures the targets and therefore attaches the PNIPAM molecule to their surface. Besides PNIPAM, a family of temperature-responsive molecules may function in the same manner.[26,27] In addition, other biopolymers, such as elastin-like polypeptides (ELPs), are also able to undergo a reversible phase transition from water-soluble forms into hydrophobic aggregates over a wide range of temperature and pH.[28−30] Such polypeptides may be fused to antibodies through protein engineering.[31−33] In particular, functional ELP fusions with ProA, ProG, or ProL are able to conjugate with a wide range of antibodies,[34−36] and thus may be used in our multistage separation processes. It is worth noting that the PS microspheres used in our experiment have similar size as cells and, therefore, our result implies that the separation method presented here may be applicable to separation and purification of cells. Certainly, one concern about using this method for biological applications is that the proteins (such as the antibodies used for targeting) may be sensitive to temperature and certain mechanical forces, which may need to be addressed for specific applications.

CONCLUSIONS

We have demonstrated a multistage magnetic separation process that is able to separate PNIPAM-functionalized PS microspheres from bare PS microspheres by using PNIPAM-functionalized MNPs. The reversible hydrophilic-to-hydrophobic transition of PNIPAM molecules enables us to manipulate the hydrophobic interactions between the MNPs and the microspheres upon cycling the temperature, and to separate the target microspheres from nontarget microspheres in multiple stages through capture-and-release cycles. The overall enrichment factor is observed to significantly increase with the number of separation stages, and reaches as high as 1.87×10^5 after 5 stages. The result implies that such a multistage separation scheme may effectively circumvent problems caused by the nonspecific interactions in magnetic separation processes.

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Notes

The authors declare no competing financial interest.

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