Rapid and Controlled Transition of Magnetic Nano- to Micro-particles: A Useful Feature for Bioseparations

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ABSTRACT

For solution-based immunoassays and bioseparations, magnetic nanoparticles have many potential advantages over commonly used magnetic microbeads. These advantages are due to high surface area to volume ratio, favorable diffusion coefficients, and better access to targets in sterically constrained environments. However, magnetic nanoparticles have poor magnetophoretic mobility, and they do not separate with simple magnets. Stimuli-responsive mNPs can circumvent this problem. These mNPs are stable colloids for rapid diffusion and binding at one condition but an appropriate stimulus causes them to transition to micron-sized, hydrophobic aggregates for easy separation. This work describes the development and characterization of stimuli-responsive mNPs. To demonstrate their utility in bioseparation assays, these mNPs are combined with similarly stimuli-responsive antibodies to isolate antigens from human serum samples.

Keywords: magnetic nanoparticles, stimuli-responsive polymers, immunoassays, RAFT polymerization

1 INTRODUCTION

Magnetic microparticles are used in many common laboratory techniques like bioseparations and molecular assays. They are also integrated in numerous FDA-approved clinical immunoassay kits (e.g., Abbott ARCHITECT HIV® Ag/Ab Combo test) for rapid separation of disease biomarkers from patient serum samples. Magnetic nanoparticles (mNPs) offer three advantages over microparticles for bioseparations. First, they have favorable diffusion coefficients and do not settle out of solution, which enables efficient interrogation of the sample. This leads to more rapid binding of target molecules. Second, they have a higher surface area:volume ratio, which enables more binding of target molecules. This leads to higher sensitivity and lower limits of detection. Third, the small mNPs do not suffer from steric hindrance effects in complex biologic milieu like tissue homogenates or near cell surface receptors, so they may be able to bind targets that are inaccessible to larger microparticles. The mNPs, however, suffer from a major drawback in that they are too small to separate with a simple magnet. Thus, mNPs have not been adopted in clinical immunoassays or other laboratory bioseparations.

We developed a reagent system (Figure 1) that combines stimuli-responsive polymers with mNPs and biomolecules [1]. Stimuli-responsive polymers change their properties (i.e., from hydrophilic to hydrophobic) in response to environmental triggers such as temperature, solution ionic strength or pH. Biomolecules [2] and nanoparticles [3] modified with these polymers exhibit similar stimuli-responsiveness. Therefore, at one condition, the diffusion, surface area:volume and size advantages of mNPs are maintained for rapid and efficient binding of target molecules. After a stimulus is applied, the mNPs and polymer-modified biomolecules co-aggregate into hydrophobic agglomerates that are large enough to be separated with a simple magnet. This reagent system overcomes the drawbacks of mNPs while maintaining the advantages of mNPs.

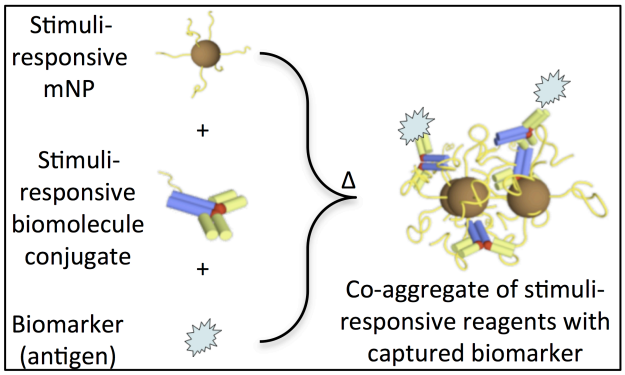

Figure 1: Schematic of stimuli-responsive binary reagent system, showing soluble mNPs, polymer-IgG conjugates and antigens at one condition. A heat stimulus causes co-aggregation of reagents, with captured biomarkers.

This work describes the development of stimuli-responsive mNPs and polymer-biomolecule conjugates, with a focus on the stimuli-responsive polymer poly(*N*-isopropylacrylamide), or pNIPAM. The mNPs and polymer-biomolecule conjugates are characterized with numerous complementary techniques. Proof-of-principle experiments show binding and isolation of HIV-1 antigen p24 from spiked human serum samples. This stimuli-responsive reagent system has applications in bioseparations and immunoassays.

2 METHODS

The polymer pNIPAM is used to make the mNPs stimuli-responsive. The pNIPAM is produced by reversible addition fragmentation chain transfer (RAFT)

polymerization, as described by others [3, 4]. The resultant pNIPAM is then further modified to enhance its thermal and chemical stablity using proprietary techniques. The stimuli-responsive mNPs are synthesized in a one-pot reaction [3], and then purified using tangential flow filtration (TFF) against distilled water. The mNPs are lyophilized and stored at 20°C until use. The stimuli-responsive polymer-antibody conjugates are synthesized and characterized as previously published [2].

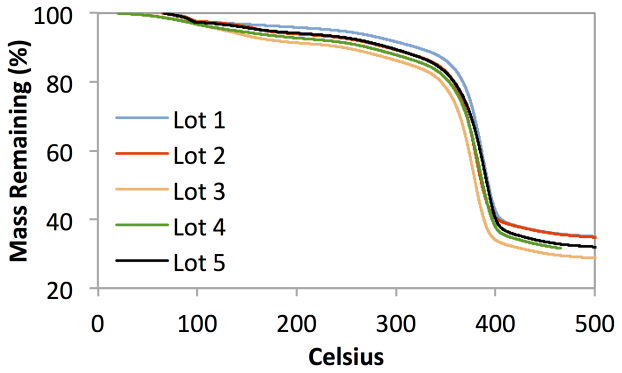

Figure 2: Thermogravimetric analysis (TGA) of 5 different lots of mNPs showing water loss at 100°C and polymer decomposition from ~250-400°C.

The mNPs are characterized with numerous techniques. The hydrodynamic size is measured by dynamic light scattering (DLS). The lower critical solution temperature (LCST) behavior is measured via UV-Visible light spectroscopy in a water-jacketed, temperature-controlled cuvette holder. Solid mNP samples are subjected to thermogravimetric analysis (TGA) to determine the polymer and iron masses. To determine the magnetic separation efficiency, mNP solutions were exposed to a magnetic field at 20°C (below the LCST) and at 37°C (above the LCST) for 2 minutes. An aliquot of the solution was then measured with UV-Vis spectroscopy to determine the mNP concentration in the tube after magnetic separation. Lastly, mNP solution temperatures were cycled around the LCST 10 times to determine the robustness of the stimuli-responsive behavior. The mNP size was measured at 20°C, the temperature was raised to 37°C over the course of ~ 2 minutes and the mNP size was measured again. The mNP solution was cooled to 20°C over the course of ~5 minutes, and the size measurement/heating cycle was repeated for a total of 18 times.

The polymer-IgG conjugates were produced and characterized as described in a previous publication [2]. To demonstrate p24 isolation from biologic samples, mNPs, polymer-IgG conjugates and p24 were mixed in 5% human serum samples. After binding for 1 hour, the solution was heated and exposed to a magnetic field at 37°C for 2 minutes each. The heating step caused mNPs and polymer-IgG conjugates to co-aggregate, thus allowing magnetic separation of the p24 bound to the polymer-IgG conjugate. The supernate was analyzed for p24 depletion with a custom ELISA. The performance of this binary reagent system was compared to commercial reagents Dynal® magnetic microparticles. The same anti-p24 IgG was linked to the surfaces of Dynal® beads, and binding efficacy was confirmed with the ELISA. The p24 depletion experiment was repeated, as described above, with the anti-p24 IgG concentration on the Dynal® beads equal to the concentration of the polymer-IgG conjugates.

3 RESULTS AND DISCUSSION

The stimuli-responsive polymer, pNIPAM, was produced by RAFT and characterized by gel permeation chromatography (M_W = 6.1 ± 0.13 kDa , PDI = 1.02 ± 0.010, n=3). This highly monodisperse polymer is typical of RAFT, and it enables very sharp transitions of stimuli-responsive behavior. The pNIPAM was further modified with proprietary techniques to enhance the chemical and thermal stability. The molecular compositions of both versions of pNIPAM were confirmed by H^1-NMR (data not shown). Although there are numerous examples of stimuli-responsive polymers, pNIPAM is the most-studied, largely because its temperature-responsive behavior is between ambient and body temperatures (~31°C) [5]. This temperature range is convenient for instrument-based assays and benchtop experiments.

The stimuli-responsive mNPs were produced in a one-pot reaction, as described previously [3]. With this system, the mNPs are immediately water-soluble. This is different from most other mNP synthesis techniques which require laborious capping or ligand exchange reactions to render the particles water-soluble. After purification by TFF, the mNPs were lyophilized for storage at room temperature. Yields are typically ~30%.

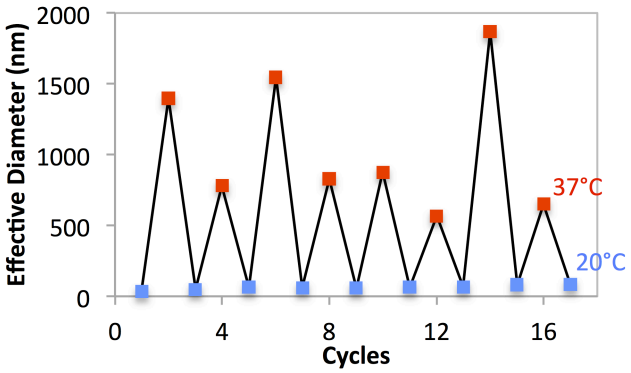

Figure 3: DLS measurements of robust mNP transition from nano-sized particles at 20°C (in blue) to micron-sized aggregates at 37°C (in red).

Every batch of mNPs is characterized with numerous techniques. First, the mNPs are measured with DLS, which shows average hydrodynamic diameters of 22.2 ± 4.23 nm (n=5). These are similar sizes to earlier work [3]. The stimuli-responsive behavior of the mNPs is measured by the LCST. This is the temperature at which temperature-

responsive polymers transition from hydrated macromolecules to hydrophobic, micron-sized aggregates. Since mNPs are coated with pNIPAM, they also exhibit LCST behavior. In fact, the mNPs display an LCST of 31 ± 0.47°C (n=5). Therefore, at room temperature (20°C), the mNPs are nano-sized and can't be separated. Above the LCST (37°C), the mNPs are aggregated and can be separated with a magnet. This separation efficieny was quantified at 20°C (4.6 ± 3.6%) and at 37°C (97 ± 0.89%), with 2 minutes allowed to both heat the mNP solution and collect with a magnet. The high separation efficiency shows that the surfaces of mNPs are coated with stimuli-responsive polymers, the mNPs self-aggregate and are removed from solution, but only at temperatures above the LCST. The separation time for these near-micron mNP aggregates is similar to that of standard microbeads; commercial suppliers of magnetic microbeads recommend 2 minute separations to completely remove their beads from solutions. The low separation efficiency at 20°C shows that the mNPs are stable colloids and too small to separate. Finally, the mNPs were subjected to TGA, which determined the relative masses of the iron oxide core and the pNIPAM corona (Figure 2). As the TGA oven temperature was increased, loosely associated water was vaporized at about 100°C, the pNIPAM corona started to decompose at about 250°C and only the iron oxide mNP core remained at 500°C. The mNPs consist of 32 ± 2.6% iron oxide (n=5), which corresponds to a polymer:iron mass ratio of 2.0 ± 0.22.

	Month	Size (nm)	Separation at 20°C	Separation at 37°C
Buffer	0	19.4	0.2%	99%
	3	22.7	0.0%	98%
Lyophilized	0	27.4	5.3%	99%
	3	26.4	0.0%	100%

Table 1: Stability studies of mNPs stored in buffer or as lyophilized powder. The mNP size (measured by DLS) and performance (measured by magnetic separation efficiency) was not affected by long-term storage.

Multiple heating and cooling steps are required in many molecular assays and bioseparations (e.g., polymerase chain reaction, or PCR). For widest adoption in the life science research market, stimuli-responsive mNPs must withstand multiple heat/cool cycles and transition between nano-dimensions at low temperatures and micron-dimensions at higher temperatures. This behavior was tested via DLS (Figure 3). At 20°C, the mNP diameters were ~ 25 nm. At 37°C, the mNPs were hydrophobic and self-aggregated into micron-sized particles, as predicted from the LCST behavior. Cooling the mNPs below the LCST allowed those aggregates to re-solubilize completely, and individual, soluble mNPs of the original size (< 50 nm) were detected by DLS. Multiple heating cycles did not alter the ability of mNPs to re-solubilize to nano-sized particles. The micron-sized mNP aggregates showed a range of sizes (~500 to 2000 nm). It is difficult to predict how neighboring mNPs interact during the aggregation process, and it leads to hydrophobic clusters of mNPs with different sizes and morphologies. Regardless, these aggregates are all easily separated with a simple magnet.

The mNPs are stable under typical storage conditions. For example, the size and stimuli-responsive performance of the mNPs barely changes after storage in buffered solutions for 3 months (Table 1). This result shows that the mNPs do not aggregate and settle out of solution over time. The lyophilized mNPs are also remarkably stable when stored at room temperature – there were no significant changes in properties or performance after 3 months (Table 1 – and data collection ongoing).

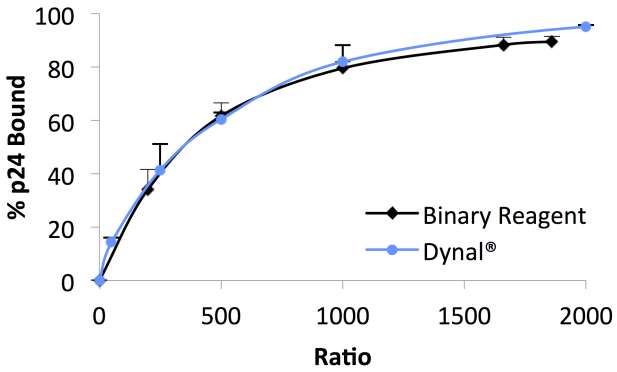

Figure 4: The stimuli-responsive binary reagent system (mNPs and polymer-IgG conjugates) bound and isolated p24 antigen from spiked human serum samples with similar binding efficacy as the commercial magnetic microparticle reagent Dynal®.

It is possible to conjugate biomolecules directly to the mNP surfaces. However, a different approach was taken here, in which another stimuli-responsive reagent was developed to work with the mNPs (Figure 1). Specifically, a larger, ~36 kDa pNIPAM was covalently conjugated via amine chemistry to anti-p24 IgG antibody molecules, as described previously [2]. These polymer-IgG conjugates bind to HIV-1 p24 protein antigen with similar binding affinity as the native anti-p24 antibody. The polymer-IgG conjugates are stimuli-responsive but with a higher LCST value of ~ 37°C. The mNPs and polymer-IgG conjugates respond to the same types of stimuli (i.e., temperature) because the pNIPAM stimuli-responsive polymer is the same on these reagents. Thus, at low temperatures both these reagents are hydrophilic and soluble, with molecular dimensions. At higher temperatures, the mNPs both self-aggregate and co-aggregate with the polymer-IgG conjugates. Therefore, p24 antigen bound to the anti-p24 IgG can be isolated from samples after polymer-IgG co-aggregation with mNPs at temperatures above the LCST (Figure 4). As a control, this experiment was run simultaneously with anti-p24-labeled Dynal® magnetic microparticles, which are commercial reagents for this type

of immunoassay. The binding and isolation efficiency of the binary reagent system is nearly identical to the Dynal® system. This experiment showed that the stimuli-responsive binary reagent system can capture protein antigens from biologic samples.

4 CONCLUSIONS

The stimuli-responsive mNPs developed in this work show useful properties for bioseparations: small size for binding and diffusion, rapid and controlled stimuli-responsive behavior, rapid magnetic separation once aggregated, stability under normal storage conditions and robust behavior with temperature cycles. The manufacturing process produces mNPs of good reproducibility and consistency. Combining these mNPs with polymer-biomolecule conjugates results in a unique binary reagent system with favorable biomarker target binding. This stimuli-responsive binary reagent system has potential applications in immunoassays and other bioseparations in the life science reasearch fields.

5 REFERENCES

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