Supporting Information

Microfluidics-enabled rational design of immunomagnetic nanomaterial and its shape effect on liquid biopsy

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Experimental details

1. Materials and reagents

Ferric chloride hexahydrate (FeCl₃·6H₂O), sodium hydroxide (NaOH), sodium sulfate (Na₂SO₄), hydrochloric acid (HCl, 37%), tetraethyl orthosilicate (TEOS), ammonium hydroxide (25%), ethanol (200-proof), 3-Aminopropyltrimethoxysilane (APTMS), N,N-dimethylformamide (DMF), N-Hydroxysulfosuccinimide (NHS), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), glutaric anhydride, Cell-counting kit-8 (CCK-8), Hoechst 33342, Tween-20, and ethanol (200-proof) were purchased from Sigma-Aldrich. Anti-Pan Cytokeratin eFluor® 615 was purchased from eBioscience. Anti-EpCAM FITC conjugate was obtained from Molecular Probes. CellSearch profile kit containing Anti-EpCAM Ferrofluid was purchased from Veridex. Polydimethylsiloxane (PDMS Sylgard 184) was purchased from Dow Corning. Water used was from a Milli-Q water ultrapure water purification system. All chemicals were used as received without any further purification.

2. Fabrication of spiral-shaped microfluidic reactors

The five-run microfluidic spiral channel having two inlets and one outlet was fabricated using polydimethylsiloxane (PDMS) through soft lithography. Briefly, after designing the pattern with AutoCAD, film mask was obtained (from Fine Line Imaging, Inc.) to fabricate the SU-8 2035 master mold using standard photolithography. PDMS replica was then produced by pouring PDMS precursor onto the mold and curing the structures at 65 °C for 1.5 hours. Microchannels were formed by bonding the PDMS replicas to standard glass slides after oxygen plasma treatment. The smallest diameter of the spiral microchannel is 5.25 mm, and then it increases from 11.0 mm to 22.2 mm with an increment of 1.4 mm for each half run. The width and the height of the microchannel are 500 μm and 50 μm, respectively.

3. Synthesis of MNPs
The synthesis of α-Fe$_2$O$_3$ MNPs was realized by simply using one fluid containing ferric chloride and the other sodium hydroxide and sodium sulfate modified from previous protocols.\textsuperscript{1–5} Specifically,

\textit{Inlet I}: FeCl$_3$·6H$_2$O was dissolved in water to make a 0.02 M solution. The mixed solution was transferred into syringe I.

\textit{Inlet II}: NaOH and Na$_2$SO$_4$ were dissolved in water to make a 0.06 M solution. The mixed solution was transferred into syringe II.

The two inlet flows, \textit{Inlet I} and \textit{Inlet II}, were pumped (Pump 33 DDS, Harvard Apparatus) into the spiral microchannel at certain flow rates to produce MNPs of different shapes. Specifically, sMNPs, cMNPs, rMNPs, and bMNPs were fabricated when the flow rates of FeCl$_3$ fluid and NaOH/Na$_2$SO$_4$ fluid were set as 250 and 100 μL/min, 50 and 50 μL/min, 100 and 40 μL/min, 10 and 25 μL/min, respectively. The collected fluids from the outlet were sealed tightly and placed in a 120 °C oven overnight. Afterward, the solids were obtained after centrifugation and thorough washing with DI water and then dried at 60 °C to produce MNPs of different shapes.

4. COMSOL simulation details and details of Reynolds number for the microreactors

Reynolds number (Re) was calculated to determine if the fluids are laminar flows: $Re = \frac{\rho UL}{\mu}$, where the density ($\rho \sim 1000$ kg/m$^3$) and dynamic viscosity ($\mu \sim 0.001$ Pa·s) of water are used for approximations, L is the characteristic length, and $U$ is the average flow velocity, which could be obtained by: $U = \frac{\text{flow rate}}{W \cdot H}$.

In our case with the spiral microchannel, $W = 500 \, \mu$m, $H = 50 \, \mu$m, flow rate $\leq 250$ μL/min, so $U = 0.0675$ m/s, and Re is less than 15.35 ($< 2300$). And when the flow rate of 100 μL/min or less is used in this study, the Re will be even smaller.

Therefore, the fluids are laminar flows. We consider them as incompressible with no-slip boundary condition, and neglect the gravity force for simplicity. The flow rates for two inlets are the corresponding
numbers used in the synthesis of differently shaped MNPs. The outlet is set to be fixed pressure with \( p = 0 \). The diffusion coefficient used is \( D = 5 \times 10^{-10} m^2/s \).

### 5. Calculation of the time for the synthesis of MNPs via microreactors

In this study, the width and height of the microchannel are 500 \( \mu \)m and 50 \( \mu \)m, respectively. The smallest diameter of the spiral microchannel is 5.25 mm, and then it increases from 11.0 mm to 22.2 mm with an increment of 1.4 mm for each half run. To estimate the time for synthesis, firstly, the length of the channel is calculated, which is 25.07 cm. Then the volume of fluids in the channel is calculated to be 6.27 \( \mu \)L. Thus, with a flow rate of 10-250 \( \mu \)L/min, the time for the synthesis of magnetic nanostructures is \(~1.50-37.6\) s.

### 6. Synthesis of MNPs@Silica

The synthesis of MNPs@Silica was realized using one fluid containing MNPs and the other TEOS. Specifically,

**Inlet I**: MNPs (1 mg/mL) were dispersed in water and then mixed with 1.33 M ammonia. The mixture was transferred into syringe I.

**Inlet II**: TEOS was dissolved in ethanol to make a 0.045 M solution. The mixed solution was transferred into syringe II.

The two inlet flows, **Inlet I** and **Inlet II**, were pumped (Pump 33 DDS, Harvard Apparatus) into the spiral microchannel at certain flow rates to produce MNPs of different shapes. The shell thickness of silica was tuned by the flow rate of TEOS. After reaction, the solution from the outlet was collected. Afterward, the solids were obtained after centrifugation and thorough washing with DI water and then dried at 60 °C to obtain MNPs@Silica. To confirm the formation of core-shell structure, the as-prepared materials were treated with HCl (2 M) at 80 °C overnight, and then investigated under TEM.

### 7. Synthesis of MNPs@Silica-EpCAM
The functionalization of FITC-conjugated Anti-EpCAM on MNPs@Silica surface was carried out according to our previously reported method. Firstly, MNPs@Silica (20 mg) were mixed with APTMS (20 μL) in DMF for 12 hours to graft amine groups on particle surface. Following by thorough washing, the resultant materials were reacted with 0.5 mM glutaric anhydride in DMF overnight to get the carboxylated particles. Then, EDC (5 mmol) and NHS (12.5 mmol) were dissolved in the carboxylated particles suspension (pH5.0 PBS). After 30 min, Anti-EpCAM FITC-conjugate was added to this solution, then the pH of the reaction system was adjusted to 7.5. This reaction lasted for 10 hours at 4 °C in the dark condition. The final solid was isolated by centrifugation and washing repeatedly at 4 °C to obtain MNPs@Silica-EpCAM. To ensure a consistent level of fluorescent intensity over particle concentration for the immunomagnetic nanomaterials of different shapes, we used the same nanoparticle-to-antibody ratio and tried to handle all samples in the same manner.

8. Cell culture and maintenance

MCF-7 cells (human breast adenocarcinoma cell line, ATCC) and MDA-MB-231 cells (human breast adenocarcinoma cell line, ATCC) were cultured in high glucose DMEM (Dulbecco’s Modified Eagle’s Medium, ATCC) supplemented with 10% FBS (ATCC) and 1% penicillin-streptomycin (Sigma) in a humidified incubator at 37 °C with 5% CO₂ and 95% air.

9. In vitro cytotoxicity evaluation of MNPs@Silica-EpCAM

The cytotoxicity of sphere-, cube-, rod-, and belt-shaped MNPs@Silica-EpCAM was evaluated using the CCK-8 viability assay. For the cytotoxicity evaluation, MCF-7 and MDA-MB-231 cells were seeded at a density of 5000 cells per well in 96-well plates. After incubating the cells with differently shaped MNPs@Silica-EpCAM at a particle concentration ranging from 0.1 to 1000 μg/mL for 24 h, 10 μL CCK-8 reagent was added to each well and incubated for 4 h. The absorbance of the resulting solution in each well was recorded at 450 nm with a microplate reader (TECAN SPARK 10M). Before reading, the plate was gently shaken on an orbital shaker for 30 s to ensure homogeneous distribution of color.
10. Cellular binding kinetic test of sMNPs@Silica-EpCAM

To optimize the treatment time of cells with immunomagnetic nanoparticles, we investigated the binding kinetics of sMNPs@Silica-EpCAM by MCF-7 and MDA-MB-231 cells. 10^5 cells per well were seeded in 6-well plates and were allowed to adhere for 24 h. After incubated with sMNPs@Silica-EpCAM (200 μg/mL) for 0, 15, 30, 60, 180, and 360 min, respectively, the treated cells were washed three times with PBS and then harvested by trypsinization. After the cells were collected, the cellular binding amount was quantitatively determined in FITC channel by flow cytometry (MACSQuant® Analyzer) and then analyzed by FlowJo software. At least ten thousands of cells were analyzed and the results were obtained in triplicate.

11. Fluorescent investigation of the interactions between cells and MNPs@Silica-EpCAM

MCF-7 and MDA-MB-231 cells (10^5 per well) were separately seeded in a 6-well plate containing cover glasses and were allowed to adhere for 24 h. After incubated with MNPs@Silica-EpCAM of different shapes (200 μg/mL) for 1 h, the cover glass containing MCF-7 or MDA-MB-231 cells were washed with PBS and was then mounted onto a glass slide. The slide was examined under a fluorescence microscope (Olympus BX51).

12. Flow cytometry measurement of the cellular binding efficiency of MNPs@Silica-EpCAM

MCF-7 and MDA-MB-231 cells (10^5 cells per well) were seeded in 6-well plates and were allowed to adhere for 24 h. After incubated with MNPs@Silica-EpCAM of different shapes (200 μg/mL) for 1 h, the treated cells were washed three times with PBS and then harvested by trypsinization. After the cells were collected, at least ten thousands of cells were acquired in FITC channel by flow cytometry (MACSQuant® Analyzer) and then analyzed by FlowJo software.

13. Screening system of CellRich™ microchip

The microchannel was made by a standard molding technique using polydimethylsiloxane (PDMS). Surfaces of PDMS chips and glass slides were bound together to form microchannel. One end of the
microchannel device was connected to a reservoir, while the other to a waste collection tube. A syringe pump was used to draw the blood sample from the reservoir through the microchannel, and the waste liquid was collected in a tube. An automatic rotational microfluidic device holder was developed to change the orientation of microfluidic device during the screening process, including the separation step and the flushing step. The screening system provided the function of rocking the reservoir to mix the blood sample while screening. Six samples could be screened at the same time to increase the screening throughput. More details can be found in our previously published literature.\textsuperscript{7–9} Based on the mathematical model we built in the previous publication,\textsuperscript{10} the microchannel was inversely placed during the separation process to achieve high capture efficiency. Three permanent magnets (Block NdFeB magnet, product of 42 MGOe, grade N42, \(3/4'' \times 1/2'' \times 7/32''\)) are placed outside the microfluidic device with alternating polarities.

14. Screening processing of blood samples spiked with cancer cells

MCF-7 and MDA-MB-231 suspensions were firstly mixed and incubated with trypsin (0.05% Trypsin-EDTA) for 5-10 minutes to break the cell clusters and to ensure the cells flow through the microchannel individually. Cells were then counted with a hemacytometer and diluted in phosphate buffered saline (PBS) to prepare a solution of approximately 2000 cells/mL. Then 25 and 50 \(\mu\text{L}\) of the above cell suspension were separately added to each 2.5 mL aliquot of blood to prepare a sample spiked with \(~50 \text{ and } ~100\) cells, respectively. The same amount of solution was also dispensed on each of three glass slides as counting controls. The number of cells actually spiked into the blood was determined by using the average of the three control slides as 100%, and then the capture rates were calculated. Normal blood samples which were not spiked with cancer cells were prepared along with the spiked ones, and all the following procedures were performed in parallel.

All blood samples were obtained from multiple healthy donors in the Dartmouth-Hitchcock Medical Center with informed consent from all participants under an IRB-approved protocol. This study was approved by the Institutional Biosafety Committee (IBC) and the Advisory Committee on Human Research at the Dartmouth College. All screening tests were performed in accordance with the declaration of Helsinki.
Whole blood samples were collected in CellSave™ tubes and were screened within 24–48 hours after collection, simulating the actual screening situation of patient blood. Before screening, the blood was processed as follows: firstly, 3.5 mL of dilution buffer solution (Veridex, LLC) is added to the above blood and the mixture was centrifuged at 800g for 10 min. Supernatant containing plasma as well as the buffer solution was removed and the buffer solution was added again to make a total of 3.5 mL of the sample. These steps replaced blood plasma with the dilution buffer. Secondly, a suspension of MNPs@Silica-EpCAM of different shapes (0.2 μg) was added to the blood. Veridex Ferrofluid conjugated with EpCAM was used as a comparison. The screening process started 60 minutes after those reagents were added.

Before the blood sample was introduced into the reservoir, the microchannel was filled with PBS to eject air bubbles. The spiked blood samples were then driven pneumatically at a flow rate of 2.5 mL/h. After the blood screening, PBS was introduced and flowed continuously until the red blood cells were not visible in the microchannel. This process removed unwanted blood cells from the bottom glass slide. The same rate as the blood flow was used for the flushing step, where typically ~4 mL of PBS was added. After flushing, 2 mL of ice-cold acetone at the same rate was introduced to the channel to fix cancer cells onto the glass slide. After being disassembled and dried completely, the bottom glass slide was stored at 4 °C until staining.

15. Immunofluorescence staining and cell identification

The experimental glass slide was rinsed with PBS and 0.1% Tween-20. 300 μL blocking buffer was added on the sample slide followed by the incubation at 37 °C for 60 minutes. The cells were then immunofluorescently stained with Anti-Pan Cytokeratin eFluor® 615 (1:100 dilution) for 2 hours in the staining solution (PBS, 0.1% Tween-20, and 1% BSA). The slide was then immersed in PBS and 0.1% Tween-20 for 5 minutes for three times. Next, the cells were stained with Hoechst 33342 (5 μg/mL) for 20 min. After immersed in PBS and 0.1% Tween-20 for 5 minutes for three times, the sample slides were observed under a fluorescence microscope (Olympus BX51) for cell identification and enumeration.
Samples from normal blood, which are not spiked were also stained side by side to perform blind observation. More details can be found in our recent publications.7–11

16. Characterization

Transmission electron microscopy (TEM) was performed on a Tecnai F20ST field emission gun (FEG) transmission electron microscope operating at an accelerating voltage of 200 kV. Scanning electron microscopy (SEM) was performed on a XL-30 field emission gun environmental scanning electron microscope. Magnetization curves of the particles were obtained from a Lakeshore model 7300 vibrating sample magnetometer (VSM).

References:


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**Figure S1.** Hysteresis curves of MNPs@Silica core-shell nanoparticles with different shapes recorded at 300 K.

**Figure S2.** Fluorescent spectra of MNPs@Silica-EpCAM with different shapes at a same particle concentration of 100 µg/mL.
Figure S3. MCF-7 (A) and MDA-MB-231 (B) cell viability after treated with MNPs@Silica-EpCAM of different shapes at a particle concentration ranging from 0.1 to 1000 µg/mL for 24 h.

Figure S4. Cellular binding kinetics of sMNPs@Silica-EpCAM by MCF-7 and MDA-MB-231 cells.
**Figure S5.** Our self-developed immunomagnetic microchip for CTCs screening. (A) Schematic drawing of the embedded microfluidic cartridge. (B) Photograph image of our CellRich™ system.

**Figure S6.** TEM images of Veridex Ferrofluid at different magnifications.