Supporting Information

Microfluidics-enabled rapid manufacturing of hierarchical silica-magnetic microflower toward enhanced circulating tumor cells screening

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Experimental details

1. Materials and reagents

Ferric chloride hexahydrate (FeCl$_3$·6H$_2$O), Cobalt chloride hexahydrate (CoCl$_2$·H$_2$O), polyvinylpyrroldione (PVP, 360k), hexadecylamine (HDA), tetraethyl orthosilicate (TEOS), sodium borohydride (NaBH$_4$), ethanol (200-proof), 3-Aminopropyltrimethoxysilane (APTMS), N,N-dimethylformamide (DMF), N-Hydroxysulfosuccinimide (NHS), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), glutaric anhydride, Bovine serum albumin (BSA), 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 (Product details: https://documents.cellsearchctc.com/pdf/e631600001/e631600001_EN.pdf). 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 silica microflower

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The synthesis of silica microflower was realized by simply using one fluid containing TEOS (0.34 M in ethanol) and HDA (0.02 M in ethanol) and the other having PVP (0.02 M in water).

The two inlet flows were then pumped (Pump 33 DDS, Harvard Apparatus) into the spiral microchannel both at a flow rate of 4 mL/min to produce silica microflower. Afterward, the as-synthesized product was collected from the outlet.

The production yield was calculated by the formulation: Yield (%) = \( \frac{W_n}{W_t} \times 100 \), where \( W_n \) is the net weight of product, and \( W_t \) is the theoretical weight of the sample calculated based on 100% conversion of TEOS to SiO\(_2\). In our model synthesis system, after collected from the outlet, the product was treated by precipitation (4000 rpm, 10 min), drying (80 °C, overnight), and calcination (gradient heating from r.t. to 600 °C, 8 hours). The results were obtained in triplicate.

### 4. Synthesis of FeCo magnetic nanoparticles

The synthesis of FeCo magnetic nanoparticles (MNPs) was realized via the modified co-precipitation method.\(^1,2\) In brief, FeCl\(_3\) \( \cdot \) 6H\(_2\)O (0.1 M) and CoCl\(_2\) \( \cdot \) H\(_2\)O (0.1 M) were successively dissolved in the purified deoxygenated water. Then NaBH\(_4\) solution (0.77 M, 10 mL) was added dropwise into the above solution under vigorous stirring. The MNPs were collected by using magnetic field and thoroughly washing with purified deoxygenated water.

### 5. Synthesis of silica-magnetic microflower (SMMF)

The synthesis protocol of SMMF was same as that of silica microflower except of adding FeCo MNPs into PVP as one inlet flow.

### 6. The Reynolds number and simulation details of the microreactors

The Reynolds number (\( Re \)) was calculated to determine if the fluids are laminar flows: \( Re = \frac{\rho UL}{\mu} \), where the density (\( \rho \sim 1000 \text{ kg/m}^3\)) and dynamic viscosity (\( \mu \sim 0.001 \text{ Pa\cdot s} \)) 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, \( W = 500 \, \mu\text{m} \), \( H = 50 \, \mu\text{m} \), flow rate = 4 mL/min, so \( U = 1.08 \, \text{m/s} \), and \( Re \) is less than 245.6 (< ~2300).

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

**7. Calculation of the time for the synthesis of SMMF via microreactors**

In this study, the width and height of the microchannel are 500 µm and 50 µm, 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 µL. Thus, with a flow rate of 4 mL/min, the time for the synthesis of magnetic nanostructures is 93.75 ms.

**8. Synthesis of SMMF-EpCAM**

The functionalization of FITC-conjugated Anti-EpCAM on SMMF surface was carried out according to our previously reported method.\(^3\) Firstly, SMMF (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 material was 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 SMMF-EpCAM. Using a similar strategy, we modified BSA-FITC conjugated on SMMF surface to yield SMMF-BSA as control.

**9. 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.

10. Cellular uptake kinetic test of SMMF-EpCAM over time

To optimize the treatment time of cells with immunomagnetic nanoparticles, we investigated the uptake kinetics of SMMF-EpCAM by MCF-7 and MDA-MB-231 cells. 10⁵ cells per well were seeded in 6-well plates and were allowed to adhere for 24 h. After incubated with SMMF-EpCAM (100 μg/mL) for 0, 5, 15, 30, 60, 120, 180, and 300 min, respectively, the treated cells were washed three times with PBS and then harvested by trypsinization. After the cells were collected, the cellular uptake 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. In vitro cytotoxicity evaluation of SMMF-EpCAM

The cytotoxicity of SMMF-EpCAM was evaluated using the CCK-8 viability assay. For 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 SMMF-EpCAM at particle concentrations ranging from 1 to 250 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.

12. Cellular binding efficiency of SMMF-EpCAM at different particle concentrations

MCF-7 and MDA-MB-231 cells (10⁵ cells per well) were seeded in 6-well plates and were allowed to adhere for 24 h. After incubated with SMMF-EpCAM of different particle concentrations (25, 100, and 250 μg/mL) for 30 min, 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. Fluorescent investigation of the interactions between cells and SMMF-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 SMMF-EpCAM (100 μg/mL) for 30 min, the cells were treated with Hoechst 33342 (10 μg/mL) for 10 min. The cover glass containing MCF-7 or MDA-MB-231 cells were washed with PBS and then mounted onto a glass slide. The slide was examined under a fluorescence microscope (Olympus BX51).

14. SEM investigation of the cellular binding efficiency of SMMF-EpCAM at different particle concentrations

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 SMMF-EpCAM of different particle concentrations (25, 100, and 250 μg/mL) for 30 min, the treated cells were washed three times with PBS and then fixed in 2.5% (w/w %) glutaraldehyde for 3 h. Finally, cell samples were dehydrated through graded ethanol solutions of 30, 50, 70, 85, 95, and 100 %, for 10 min each. The samples were then dried and investigated under SEM.

15. 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{4-6} Based on the mathematical model we built in the previous publication,\textsuperscript{7} 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 alternate polarities.

16. Screening of blood samples spiked with cancer cells

MCF-7 and MDA-MB-231 suspension 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 hemocytometer and diluted in phosphate buffered saline (PBS) to prepare a solution of approximately 2000 cells/mL. Then 25 $\mu$L of the above cell suspension was added to 2 mL aliquot of blood to prepare a sample spiked with ~50 cells. 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.

Blood samples were drawn from multiple healthy donors after obtaining informed consent under an IRB-approved protocol. All screening tests were performed in accordance with the declaration of Helsinki. Whole blood samples were collected in CellSave\textsuperscript{TM} 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, 2 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 2 mL of the sample. These steps replaced blood plasma with the dilution buffer. Secondly, a suspension of SMMF-EpCAM (0.1 $\mu$g) was added to the blood. Veridex Ferrofluid conjugated with EpCAM (Product details:
https://documents.cellsearchctc.com/pdf/e631600001/e631600001_EN.pdf) and SMMF-BSA were used as a comparison. The screening process started 30 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 flow rates of 2.5 and 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 2-4 mL of PBS was added. After flushing, 1-2 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.

For SEM investigations, additional fixation treatments with 2.5% glutaraldehyde and graded ethanol solution treatment (30, 50, 70, 85, 95, and 100%, 10 min each) were administered.

17. 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 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 (10 µg/mL) for 10 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.4–8

18. 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:
Figure S1. Schematic of the fabrication process of microfluidic reactor used in this study.
Figure S2. SEM images of the product prepared using the same protocol as silica microflower but without PVP.

Figure S3. SEM images of the product prepared using the same protocol as silica microflower except of increasing the flow rate from 4 mL/min to 6 mL/min.
Figure S4. TEM image of the as-synthesized FeCo nanoparticles.

Figure S5. Hysteresis curves of FeCo magnetic nanoparticles recorded at 300 K.
**Figure S6.** Hysteresis curves of silica-magnetic microflower recorded at 300 K.

**Figure S7.** Photograph of magnetic separation behaviour under an external magnet.
**Figure S8.** Fluorescent spectra of SMMF-EpCAM showed an obvious FITC peak, demonstrating the successful antibody conjugation.

**Figure S9.** MCF-7 and MDA-MB-231 cell viability after treated with SMMF-EpCAM at particle concentrations ranging from 1 to 250 µg/mL for 24 h.
Figure S10. Our self-developed immunomagnetic microchip for CTCs screening. (A) Schematic drawing of the embedded microfluidic cartridge. (B) Photograph of our CellRich™ system.

Figure S11. TEM images of Veridex Ferrofluid.
Figure S12. The representative SEM images of SMMF-BSA/Veridex Ferrofluid captured MCF-7 cell and MDA-MB-231 cell. Scale bar=5 μm.