Supporting Information

Development of Multifunctional Fluorescent-Magneto Nanoprobes for Selective Capturing and Multicolor Imaging of Heterogeneous Circulating Tumor Cells

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Methods

Polyethyleneimine (PEI, 25 kD), D,L-Lactide (DLLA), polyvinyl alcohol (PVA), graphite, KMnO4, nitric acid, ethylene glycol, FeCl3, sodium borohydride, sodium acetate, and 1,6-hexadiamine, antibody and all other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). All epithelial, mesenchymal, stem and normal cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD).

1. Synthesis and Characterization

Figure S1: A) Schematic representation showing the synthetic route we have used for the development of blue fluorescence polymer dots. B) Schematic representation showing the synthetic route we have used for the development of polymer dots attached fluorescent-magnetic nanoplatform (magneto-PDs nanoprobes). C) TEM image shows the morphology of PDs. Inserted HRTEM image indicate that the particle size is around 4 nm. D) SEM image shows the morphology of carboxy acid functionalized magnetic nanoparticle. Inserted Energy-dispersive X-ray (EDX) spectroscopy elemental image shows he presence of Fe in magnetic nanoparticle. E) SEM image shows the morphology of magneto-PDs nanoprobe. Inserted EDX elemental mapping shows the presence of Fe, C and O in fluorescent-magnetic nanoplatform, F) Figure shows the histogram of size distribution for polymer dots measured by DLS.

Table S1: Average size for the synthesized polymer dots, magnetic nanoparticle and magneto-PDs nanoprobe, measured by DLS and SEM.

| Nanoparticle description | Size measured by DLS | Size measured by SEM |
|--------------------------|----------------------|---------------------|
| Polymer dots             | 3 ± 2 nm             | 3 ± 1 nm            |
| Magnetic nanoparticle    | 30 ± 8 nm            | 30 ± 6 nm           |
| Magneto-PDs nanoprobe    | 40 ± 12 nm           | 40 ± 10 nm          |

1.1. Synthesis of blue fluorescence polymer dots

Blue fluorescence polymer dots (PDs) were synthesized using amphiphilic polymer solvent evaporation technique. For this purpose, amphiphilic copolymer was constructed by conjugating polyethyleneimine and D,L-lactide using ring-opening polymerization method, as shown in Figure 1A. 15 g of pre-dehydrated DLLA was dissolved by stirring in 50mL of anhydrous dimethysulfoxide solvent. To this solution 0.5g of PEI is added. After PEI is dissolved, 0.05M trimethylamine is added. The obtained solution was kept under nitrogen at 86°C under gentle stirring. After 12h the solution was poured on ice cold water and the precipitate collected and was thoroughly washed with nanopure water. The product was ex-
tracted using toluene. In the next step, for the development of polymer dots. For this purpose, 20mg of PEI-PLA copolymer was dissolved in 2mL of dichloromethane. This solution was added to 1% (w/v) of PVA. The resulting mixture was sonicated until clear solution is obtained. The clear solution was kept at 35°C in vacuum chamber to let the dichloromethane evaporate. The resulted aqueous solution was centrifuged at 1500 rpm for 20 minutes and thoroughly washed to remove any unreacted reactants. The purified particles were characterized by high-resolution SEM, TEM and DLS measurement, as shown in Figure 1 and table 1. Figure S1C shows the TEM image of polymer dots. Inserted high-resolution image shows that the size of polymer dots is about 2-3 nm. Since it is well known that TEM grid preparation can increase aggregation, as we have noted in our TEM image reported in Figure S1C, we have also performed dynamic light scattering (DLS) measurement using a Malvern Zetasizer Nano instrument in solution phase. Both DLS and SEM data, as reported in Table S1, indicate that the average size is about 3 nm for polymer dots.

1.2. Synthesis of acid functionalized magnetic nanoparticle: carboxy acid functionalized magnetic nanoparticle were prepared from ferric chloride and 1,6-hexanedioic acid using co-precipitation method as shown in Figure S1A. For this purpose, 2.703 g (10 mmol) of FeCl$_3$.6H$_2$O and 0.994 g (4.24 mmol) of FeCl$_2$.6H$_2$O were dissolved in 50 ml of water. The mixture was kept under nitrogen atmosphere and heated at 80°C in oil bath with constant stirring for half an hour. Then 10 ml of 20% ammonia solution was added slowly into the reaction mixture and kept for another half an hour. Then 2 ml aqueous solution of 1,6-Hexanedioic acid (0.37 gm/ml) was added slowly to the reaction mixture. Then the solution was continuously refluxed at 200°C for six hours. The obtained black precipitate of Fe$_3$O$_4$ nanoparticles were then thoroughly washed with water for several times and separated from supernatant using neodymium magnet. Nanoparticles were characterized high-resolution SEM, TEM and DLS measurement, as reported Figure S1 and Table 1. As shown in Figure 1D, high-resolution SEM image shows that the average particle size is about ~30 nm. DLS measurement, as reported in Table 1 also indicate that the average size is about 30 nm for magnetic nanoparticle. Inserted Energy-Dispersive X-ray (EDX) spectroscopy elemental mapping in Figure 1D clearly shows the presence of Fe in the developed magnetic nanoparticle.

Figure S2: A) Schematic representation showing the synthetic route we have used for the development of red fluorescence gold cluster dots (GCDs) attached magnetic nanoprobes (magneto-GCDs nanoprobes). B) SEM image shows the morphology of amino functionalized magnetic nanoparticle. Inserted EDX elemental mapping shows the presence of Fe. C) FTIR spectra from magneto-GCDs nanoprobes verifies the existence of different amide bands. D) Figure shows the histogram of size distribution for gold dots measured by DLS. E)
TEM image shows the morphology of amino functionalized magnetic nanoparticle.

Table S2: Average size for the synthesized GCDs, amine functionalized magnetic nanoparticle and magneto-GCDs particle, measured by DLS and SEM

| Nanoparticle description | Size measured by DLS | Size measured by TEM/SEM |
|--------------------------|----------------------|--------------------------|
| Gold cluster dots        | 3 ± 2 nm             | 4 ± 2 nm                 |
| Magnetic nanoparticle    | 40± 7 nm             | 40± 5 nm                 |
| Magneto-GCDs nanoprobe   | 58± 10 nm            | 55± 10 nm                |

1.3. Synthesis of blue fluorescence magneto-PDs nanoparticles: We have used EDC/NHS esterification to produce PDs coated magnetic nanoprobe, as shown in Figure S1A. For this purpose, 10 ml (1mg/ml) of water dispersed polymer Dots was mixed with 50 ml (2 mg/ml) acid functionalized Fe₃O₄ nanoparticles in 100 ml beaker at room temperature. Then 2 ml of 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC, 1mg/ml), 500 µl N-hydroxysulfosuccinimide (NHS, 1mg/ml) and 100 µl of 4-(Dimethylamino) pyridine (DMAP, 1mg/ml) were added in the reaction mixture with constant stirring for 24 hours. Then esterified PDs attached Fe₃O₄ nanoparticles were separated using neodymium magnet by several times washed with distilled water to remove the excess reactants and stored at 4°C. The purified magnetato-PDs nanoparticles were characterized by high-resolution SEM, TEM and DLS, as reported in Figure S1 and Table S1.

1.4. Synthesis of red fluorescence GNCs: 0.2ml Sodium hydroxide (2M) was diluted to a volume of 80.2 ml in the 250 ml conical flask using pure distilled water. 26 mg of α-Lipoic acid (10.0 µM) was dissolved in this solution at room temperature under vigorous stirring. 0.8ml of HAuCl₄·3H₂O (50.78mM) was slowly added drop wise and followed the gradual addition of 800 ul of 100mM NaBH₄ with constant stirring. The resulting solution was heated for 4 minutes with a domestic microwave (900W). The initial yellow solution turned dark pink color following heating and was allowed to cool to room temperature. The obtained GNCs were purified by centrifugation at 7500 rpm for 45 minutes using a filter with 3,000 MWCO. The solid residue was collected in a 20 ml scintillation vial, dilute to a final volume of 5 mL with distilled water, and stored at 4°C for future use. The purified GCDs were characterized by high-resolution SEM, TEM and DLS measurement, as reported in Figure S2, Figure 2 and Table S2.

1.5. Synthesis of amine functionalized magnetic nanoparticle: Amine functionalized magnetic nanoparticles were synthesized by dissolving 1.5 g FeCl₃ in ethylene glycol, as we have reported before. After that we have added 2.0 g anhydrous sodium acetate. In the next step, 4.0 g of 1,6-hexadiamine was added and stirred vigorously to acquire a transparent solution. The mixture was sealed in a teflon-lined stainless steel autoclave and was heated at 230 °C for 8 h and then was washed with hot water and ethanol. At the end, the product was dried at 50 °C to get the black powder. Figure S2B and S2E shows the SEM and TEM image of amine functionalized magnetic nanoparticles, which indicate the particle size is about 40 nm. Inserted EDX mapping in Figure 2D clearly shows the presence of Fe.

1.6. Synthesis of red fluorescence magneto-GCDs nanoparticles: In the final step we have synthesized red fluorescence magneto-GCDs nanoparticles. For the formation of fluorescent-magnetic nanoparticles, we have used coupling chemistry between -CO₂H group of α-Lipoic acid attached GCDs and -NH₂ group of amine-functionalized magnetic nanoparticle via amide linkages, as shown in Figure 2A. Synthetic details has been described in the supporting information. In brief, after vortexing, 1 ml of 2 mg/ml 1-(3(dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) was added to the reaction mixture of GCDs and magnetic nanoparticle. After 20 minutes, 5 ml of Fe₃O₄-NH₂-C₆H₁₂-NH₂ nanoparticles were added drop wise with constant shaking. The mixture was sonicated for 4 hours and kept overnight under shaking and then dialyzed overnight in a 2000 Dalton MWCO dialysis tube. The purified particles were characterized by various spectroscopic techniques like Fourier transform infrared spectroscopy (FTIR), TEM and EDX analysis, as reported in Figure S2 and Figure 2.
1.7. Hydrothermal Synthesis of green fluorescence carbon dots (CDs) using ortho phenylenediamine: 0.180 g of ortho-phenylenediamine was dissolved in 180 ml pure ethanol and then the solution was transferred into a stainless steel autoclave with a teflon liner and heated at 180°C for 12 hours. The autoclave was cooled to room temperature and the reaction mixture was evaporated using rotary evaporator. The orange color carbon dots were further purified with a silica column chromatography using mixtures of CH₂Cl₂ and MeOH as eluents. Yield: 0.018g, 10%. Fig. 3B shows the TEM image of freshly prepared CDs which are about 8 nm size. DLS measurement in Table S3 indicates that the average size is about 10 nm for GCDs.

1.8. Synthesis of green fluorescence magneto-CDs nanoprobes: For the formation of green fluorescence magneto-CDs nanoprobes, we have used coupling chemistry between -CO₂H group of magnetic nanoparticle and -NH₂ group of CDs via amide linkages, as shown in Figure 3A. Synthetic details has been described in the supporting information. In brief, 1.5 ml of 2 mg/ml 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) was added to the reaction mixture of CDs and acid functionalized magnetic nanoparticle. After 20 minutes, 7 ml of Fe₃O₄-NH₂-C₆H₁₂-NH₂ nanoparticles were added drop-wise with constant shaking. The mixture was sonicated for 4 hours and kept overnight under shaking and then dialyzed overnight in a 2000 Dalton MWCO dialysis tube. The purified particles were characterized by SEM and EDX analysis, as reported in Figure 3. The high-resolution SEM data, as shown in Figure 3, shows the size of CDs coated magnetic nanoplateform is about 55 nm.

2. Development of Antibody Conjugated fluorescent-magnetic nanoprobes: For targeted capture and imaging of SK-BR-3 epithelial cancer cell, blue fluorescence magneto-PDs nanoprobes were attached with anti-HER2 antibody epithelial markers. To accomplish this, magneto-PDs nanoprobes were coated by amine modified polyethylene glycol (NH₂-PEG) initially. After PEGylation, anti-HER2 antibody was attached with amine functionalized PEG coated PDs attached magnetic nanoplateform using glutaraldehyde spacer method, as we have reported before 9-10,24. Similarly, to capture CAL-120 breast cancer cell having high levels of mesenchymal markers, green fluorescence magneto-CDs nanoprobes were attached with mesenchymal markers (anti- twist antibody). Also to target CSC bone marrow CD34+ stem cells, red fluorescence magneto-GCDs nanoprobes were attached with anti-CD34 antibody CSC markers.

3. Cell culture and incubation with multifunctional fluorescent-magnetic nanoprobes: Human normal skin HaCaT cell lines, breast cancer SK-BR-3 epithelial cancer cell line, CAL-120 breast cancer cell line having high levels of mesenchymal markers and bone marrow CD34+ stem cell lines were purchased from the American Type Culture Collection (ATCC) and grown according to the ATCC procedure. Once the

Table S3: Average size for the synthesized carbon dots, acid functionalized magnetic nanoparticle and magneto-CDs nanoprobe, measured by DLS and SEM.

| Nanoparticle description | Size measured by DLS | Size measured by SEM |
|--------------------------|-----------------------|----------------------|
| Carbon dots              | 10 ± 3 nm             | 9 ± 2 nm             |
| Magnetic nanoparticle    | 30± 7 nm              | 30± 5 nm             |
| Magneto-CDs nanoprobe    | 55± 8 nm              | 55± 7 nm             |
culture was more than $10^5$ cells/mL, different numbers of individual or mixture of epithelial, mesenchymal and stem cells, were spiked in citrated whole rabbit blood at various densities. Next, different concentrations of antibody attached multifunctional fluorescent magnetic nanoprobes were mixed with spiked blood for 30 minutes at room temperature before performing the magnetic separation experiment.

4. Separation of epithelial, mesenchymal and cancer stem cells from spiked blood using multifunctional magneto-fluorescent nanoprobes: To demonstrate the possible capturing of breast cancer SK-BR-3 epithelial cancer cell, CAL-120 breast cancer cell having high levels of mesenchymal markers and bone marrow CD34+ stem cells in a more physiologically relevant system, different concentrations of tumor cells and $10^6$ cells/mL peripheral blood mononuclear cells (PBMC) were spiked into 15 mL suspensions of citrated whole rabbit blood purchased from Colorado Serum Company. Using ELISA analysis, we find no HER2, EpCAM, vimentin, twist or CD34+ present in whole rabbit blood. For control experiment, citrated whole rabbit blood was spiked by HaCaT normal skin cells. Using ELISA analysis we find no HER2, EpCAM, vimentin, twist or CD34+ present in HaCaT cells. We have kept the concentration of each cell in the mixture in such a way that after mixing, the tumor cell concentration is 10 Cells/mL. After 30 minutes of gentle shaking spiked blood with antibody-attached nanoprobes, we have separated targeted cells bound magnetic nanoplateform using a bar magnet and analyzed them using ELISA kits, and fluorescence mapping as shown in Figures 4 and 5.

5. ELISA Assay to find capture efficiency for epithelial, mesenchymal and stem cells selectively: Amount of HER2, EpCAM, twist or CD34+ present levels were measured using the ELISA kit in accordance with the manufacturer's instructions. We have purchased these kits from Anogen, USCN Life Science Inc and Oncogene Science.

6. Fluorescence mapping of epithelial, mesenchymal and stem cells selectively and simultaneously: For the fluorescence mapping of epithelial, mesenchymal and stem cells selectively and simultaneously an Olympus IX71 inverted confocal fluorescence microscope fitted with a SPOT Insight digital camera was used.

7. Cell viability assay: To study the cytotoxicity, epithelial, mesenchymal and stem cells were treated with fluorescent-magnetic nanoprobes at different time intervals, and the cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The absorbance at 540 nm was recorded using Multiskan Ascent Plate Reader with the ASCENT software (Labsystems) as we have reported before.9,10,24