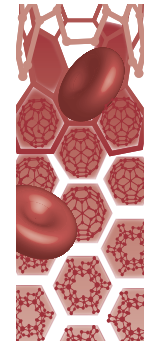
Review

Nanomedicine



Nanotechnology for enrichment and detection of circulating tumor cells

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Circulating tumor cells (CTCs) are a hallmark of invasive behavior of cancer, responsible for the development of metastasis. Their detection and analysis have significant impacts in cancer biology and clinical practice. However, CTCs are rare events and contain heterogeneous subpopulations, requiring highly sensitive and specific techniques to identify and capture CTCs with high efficiency. Nanotechnology shows strong promises for CTC enrichment and detection owning to the unique structural and functional properties of nanoscale materials. In this review, we discuss the CTC enrichment and detection technologies based on a variety of functional nanosystems and nanostructured substrates, with the goal to highlight the role of nanotechnology in the advancement of basic and clinical CTC research.

Keywords: circulating tumor cells • detection • enrichment • functional nanoparticles • microfluidics • nanostructured substrates • nanotechnology

Metastasis is the major cause of death in cancer patients, accounting for about 90% of the mortality. Although the mechanism of metastasis is not fully understood, it is known that a mandatory step of the metastatic cascade is the transport of tumor cells that are shed from the primary tumor site throughout the bloodstream of cancer patients [1]. During transport, a small population (<0.01%) of these circulating tumor cells (CTCs) arrests in a capillary bed at a distant site where they extravagate and seed the growth of a secondary tumor. The clinical value of CTC detection remains to be learned, but many studies have shown their great potential [2]. It has been realized that detection and characterization of CTCs may provide a noninvasive liquid biopsy for characterizing and monitoring cancer [3]. The prognostic significance of CTC detection has been demonstrated in several types of cancers including breast, prostate, colon, melanoma and lung cancer [4–8]. CTCs are also useful in monitoring and predicting the response to ongoing therapy [9–11]. In addition, detection of CTCs shows strong promise for early cancer detection since they have been found in blood during early stages of tumorigenesis [12]. Furthermore, molecular profiling of CTCs may offer insights into mechanisms of cancer progression and provide new therapeutic targets [13].

CTC detection and analysis, however, are very challenging [14]. The major challenge is that CTCs are rare events, as few as one CTC mixed with about 10 million white blood cells (WBCs) and 5 billion red blood cells (RBCs) in 1 ml of blood of metastatic patients [15]. Another key challenge is that they are heterogeneous in population due to tumor heterogeneity and potential changes of molecular characteristics during the epithelial-to-mesenchymal transition (EMT) [16]. As a result, significant advancement in this area has only been made in the last two decades, even though CTCs were first discovered in 1869 [17]. To date, a vast number of isolation and detection techniques have been developed, with about 100 companies offering CTC-related products and devices and over 400 clinical trials ongoing [18]. However, there is only one technique that has been approved by The US FDA for clinical

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utilization, the CellSearch system (Veridex, LLC) [15]. This technique is used to enumerate CTCs in patients with metastatic breast, prostate and colorectal cancer to help inform clinical decision making [6].

During the last two decades, the tremendous development of nanotechnology has led to the generation of a variety of advanced nanoscale materials, including metal, metal oxide, semiconductor and polymeric nanomaterials with a vast range of applications, such as in medicine [19], energy conversion and storage [20], electronics [21] and catalysis [22]. The fantasy of nanomaterials is driven by their exceptional structural and functional properties that are often not available from either bulk materials or discrete molecules due to the nanoscale effects. For example, gold nanoparticles (Au NPs) with near infrared absorption such as Au nanoshells, nanorods, nanocages and hollow nanospheres show about million times higher absorption coefficient than organic light-absorbing dye molecules, making them a new generation of photothermal agents to ablate tumor [23–27]. For CTC enrichment and detection, nanomaterials have been playing an important role, with boosted interests in recent years. The rationale is that when nanomaterials are linked with targeting ligands, they can recognize CTCs with high specificity, allowing for isolation, detection and characterization using the functional properties of the nanomaterials. In addition, nanomaterials have large surface-to-volume ratio, which enables highly efficient cellular binding in the complex blood matrix. Furthermore, nanomaterials can be readily manipulated to allow multiplexed detection and analysis, which are very important to address the heterogeneous problem of CTCs. In this review, we discuss recent progress on CTC enrichment and detection approaches using various nanoplatforms, with the goal to highlight the role of nanotechnology in the advancement of basic and clinical CTC research. These techniques are classified as *ex vivo* and *in vivo* methods, with the former category composed of enrichment, detection and emerging dual enrichment and detection methods (Figure 1).

CTC enrichment

Due to the rarity of CTCs, an initial step is often needed to isolate and enrich the tumor cells from blood cells. Current enrichment methods can be divided into two categories: those based on physical properties such as size, density and deformability, and those based on biological properties such as protein expressions [28]. Classic approaches in the former category are density gradient centrifugation, membrane filtration and microchip-based capture platforms. Approaches in the latter category include magnetic separation, substrate- and microchip-based capture platforms. The most commonly used marker for CTC recognition is EpCAM. Since epithelial cells are not usually found in circulation, the findings of EpCAM-positive cells indicate the presence of CTCs.

Magnetic nanoparticles

Magnetic nanoparticles (MNPs), commonly composed of magnetic elements such as iron (Fe) and cobalt (Co), show alignment of their magnetic moment in the presence of magnetic field. This magnetic alignment eventually pins down in the same direction of the external magnetic field under saturation [29]. Depending on the particle size, shape and composition, the magnetic response can be ferromagnetic or superparamagnetic (Figure 2A) [30]. Ferromagnetic NPs show a remnant magnetization after removal of the field, while superparamagnetic NPs do not have a remnant magnetization due to thermal fluctuations. The magnetic response causes the movement of the NPs in the direction of applied magnetic gradient and thus the MNPs can be separated from the resting solution.

Magnetic separation using magnetic particles is one of the leading CTC enrichment methods [31]. This method is easy to manipulate and exhibits high capture efficiency and specificity. Captured cells can be easily recovered by removing the magnetic field. The particles can be either microbeads (>0.5 μm) that are generally made of polymeric matrix with embedded magnetic materials, or MNPs (5–200 nm). MNPs have several distinct advantages over microbeads. They have higher cellular binding capability and excellent stability in whole blood. The small size of NPs allows the ability to attach many NPs to a cell without cell aggregation resulting in higher magnetic susceptibility (Figure 2B) [32]. Furthermore, the NPs allow for multiplexed detection by using different sized NPs or NPs labeled with different detection tags.

Bulk magnetic separation

Classical magnetic separation is done with an external permanent magnet, usually neodymium-iron-boron (NdFeB) magnet, to separate MNP-bound CTCs in a bulk solution under a stationary condition (Figure 2C). Since the magnetic force is proportional to the number of bound NPs [33], the NP-bound cells are isolated much faster than free NPs in a solution under the same magnetic field and thereby selectively enriched. The FDA-approved CellSearch system uses this approach to enrich CTCs by using 120–200 nm Fe NPs (ferrofluid) linked with anti-EpCAM antibodies [34]. In combination with immunofluroescence detection targeting cytokeratin, the system reaches over 80 recovery rate of spiked breast cancer cells [35]. Although the CellSearch system is currently the gold

standard to detect CTCs, a major limitation is that it only captures and detects EpCAM-positive cells. EpCAM expression is often heterogeneous in cancer cells and its downregulation has also been correlated with CTCs in peripheral blood [16]. This may explain why up to 70% patients known to have metastatic disease failed to exhibit detectable CTCs using the CellSearch system [36]. Another commercialized technique that uses magnetic enrichment is AdnaTest (AdnaGen AG, Germany) [37]. This technique uses a dual-capture assay, in which CTCs are captured with a mixture of large microbeads (4.5 µm superparamagnetic Dynabeads®) linked with one of two different antibodies: one against EpCAM, and the other against tumor marker such as MUC-1 or HER2 depending on the type of cancer. Subsequent detection is done with multiplexed reverse transcriptase polymerase chain reaction (RT-PCR) to recognize tumor associated mRNAs. Compared with the CellSearch system, this method improves the enrichment step by capturing CTCs with expression of any one of the two antigens. But, it is not clear whether it outperforms the CellSearch system, due to the difference in the size of the magnetic particles and the detection methods [38,39]. Different from CellSearch and AdnaTest, the commercialized magnetic activated cell sorting (MACS) technique traps CTCs labeled with superparamagnetic Fe NPs (~30 nm) within a magnetized steelwool column [40,41]. When the column is removed from the external magnetic field, the trapped cells are no longer bound to the steelwool and eluted from the column with a buffered solution. A new magnetic separation strategy that can get rid of nonspecifically labeled cells was developed by Talasaz *et al.* in 2009, which is named MagSweeper [42]. The MagSweeper uses magnetic neodymium rods to capture tumor cells labeled with anti-EpCAM linked Dynabeads in a circular loop motion. Due to the use of multiple magnetic rods and the enrichment in a motion mode that avoids nonspecifically bound species, the separator can enrich CTCs by 108-fold with 100% purity.

While the positive selection is appealing, a drawback of this method is that CTCs with low or no expression of the targeted makers can not be captured. This problem can be avoided by using negative depletion with immunomagnetic beads [43]. A general approach for negative selection with magnetic separation is to firstly lyse RBCs and then use MNPs coated with anti-CD45 antibodies to separate WBCs [44–48]. As demonstrated by Yang *et al.*, this method can reduce the number of normal blood cells by 106-fold [44]. It gives a recovery of approximately 83% on spiked head and neck cancer cells. However, due to the huge amount of normal blood cells, it is very challenging to deplete all of these background cells. In addition, lysis of RBCs may also cause loss and damage of the rare tumor cells

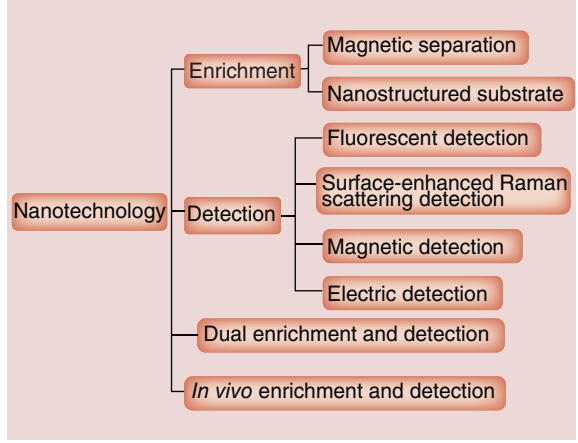

Figure 1. Nanotechnology applications in circulating tumor cells enrichment and detection.

Microchip-based magnetic separation

Microfluidic devices have become one of the mainstream platforms for CTC enrichment and detection due to many advantages including miniaturization, portability, cost–effectiveness and the abilities of online isolation/detection and single cell analysis [49]. Numerous microchip platforms have been developed based on affinity, size or other physical properties [50]. A new direction in immunomagnetic separation is to perform the separation on a microfluidic device, due to the benefits of both immunomagnetic separation and microfluidic device. Under the flow condition, the capture efficiency depends on the ratio of magnetic force and drag force [51]. A cell with many bound NPs has a large ratio of the two forces than free NPs and can thus be selectively captured.

Cell isolation in the microfluidic channels is often simply performed with permanents magnets under the chip (Figure 2D) [51–57]. The capture efficiency and sample throughput can be precisely controlled through the design of the fluidic channels, the control of the flow rate, and the magnetic field strength. Zhang and coauthors have extensively studied both experimentally and theoretically the effects of these parameters on cell capturing efficiency [51,55,56]. They showed that over 85% spiked cancer cells in blood can be captured with EpCAM targeted Fe3O4 NPs at a speed of 10 ml/h using NdFeB block magnets with a maximum energy product of 42 MGOe [55]. They also showed that the performance of the separation can be improved by inverting the microchannel (magnet placed on top of the channel) (Figure 2E) [56]. In this mode, the direction of the gravity is opposite to that of the magnetic field force. Thus, the effects of RBC sedimentation on CTC capture is greatly reduced. Using the inverted

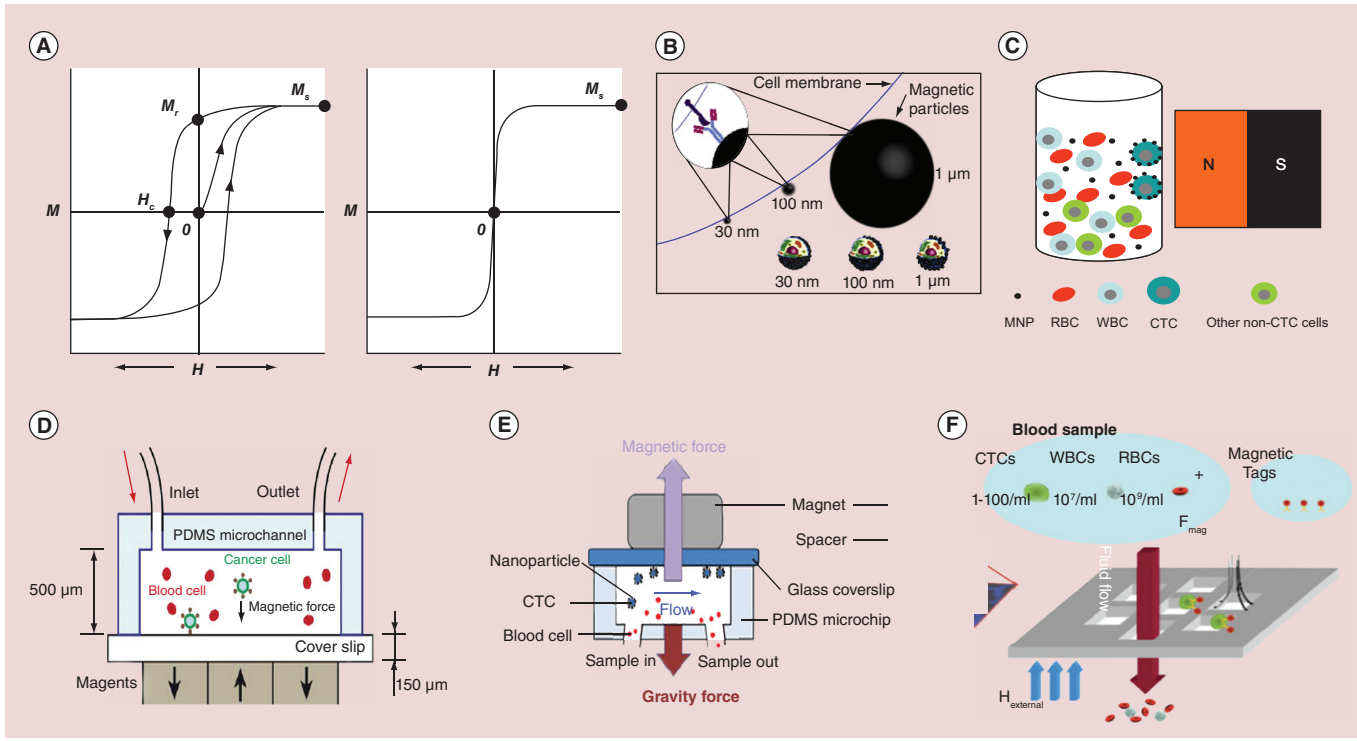

Figure 2. Magnetic nanoparticles for magnetic enrichment of circulating tumor cells. (A) Ferromagnetic and superparamagnetic properties of MNPs. **(B)** Immunomagnetically labeled cell with particles of different diameters. **(C)** Bulk magnetic separation under a stationary condition. **(D)** The principle of microchip-based immunomagnetic enrichment in an upright mode. **(E)** The principle of microchip-based immunomagnetic enrichment in an inverted mode. **(F)** Magnetic shifter device comprising an array of magnetic pores for magnetic CTC filtration.
CTC: Circulating tumor cell; MNP: Magnetic nanoparticle; PDMS: Polydimethylsiloxane; RBC: Red blood cell; WBC: White blood cell.
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microchip-based immunomagnetic separation with Fe3O4-Au core-shell NPs, Sokolov and co-authors demonstrated that CTC capture efficiency can be markedly improved by duplex targeting [57].

In some devices, the microfluidic channels are structurally designed to facilitate or enhance cell capture [58–61]. For example, the device reported by Chen *et al.* contains an array of magnetic microposts fabricated inside the channel to generate a strong magnetic force when magnetized by the external permanent magnet [59]. Validation studies showed that the device can capture 90% spiked tumor cells labeled with anti-EpCAM conjugated ferrofluid NPs at a flow rate of 6 ml/h with anti-EpCAM conjugated Fe NPs. To improve sample throughput and capture efficiency, Earhart *et al.* recently developed a magnetic shifter device, composed of an array of 40 µm holes in a silicon nitride membrane and a 12 µm thick coating of a magnetically soft permalloy (Figure 2F) [62]. Due to the extremely high field gradients at the pore edges and high density of pores (approximately 200 pores/mm^2), the magnetic microfilter combined with anti-EpCAM conjugated MNPs, can capture 96% spiked cancer cells in blood at a flow rate of 10 ml/h.

Nanostructured substrates

The past few years have witnessed the emergence of nanostructured substrates as a new platform for capturing and enriching CTCs [63,64]. When the substrates are functionalized with targeting ligands, CTCs are captured on the substrate through ligand-antigen binding. Compared to flat substrates, the major advantage of the nanostructured ones is the enhanced local topographic interactions between the substrates and targeting cell surface, which results in vastly enhanced cell capture affinity (Figure 3A) [65]. In addition, nanostructures can be coated with ligands with much higher densities than flat surfaces and thus can introduce multivalent effects to improve binding affinity. Furthermore, when the nanostructures are embedded into a microfluidic device, they lower the rolling velocity of cells in microfluidic channels and thus further enhance cellular binding.

Different types of nanostructured substrates have been reported for CTC enrichment, including nanoarray [65–71], nanofiber [72,73], nanosheet [74,75], deposited NP substrates [76–78] roughened surface [79,80] and nanoporous substrates [81,82]. The integrated NanoVel-

cro device, which has been developed by Tseng and co-authors through a combination of anti-EpCAM coated vertically oriented silicon nanopillars with vastly enhanced CTC-capture affinity and an overlaid microfluidic chaotic mixing chip capable of promoting cell-substrate contact frequency, is very promising (Figure 3B) [66]. The spiking experiments demonstrated that it can capture over 95% tumor cells. Studies on patient samples showed that the device captured significantly greater number of CTCs compared with the CellSearch system. A similar device was later developed by the same group, but with horizontally distributed poly(lactic-co-glycolic acid) (PLGA)-nanofiber as the embedded materials [73]. To facilitate the release of the tumor cells after capture, they later coated the nanoarray surface with thermal responsive polymer, poly (N-isopropylacrylamide) (PIPAAm) to manipulate cell release by changing temperature [69]. The new

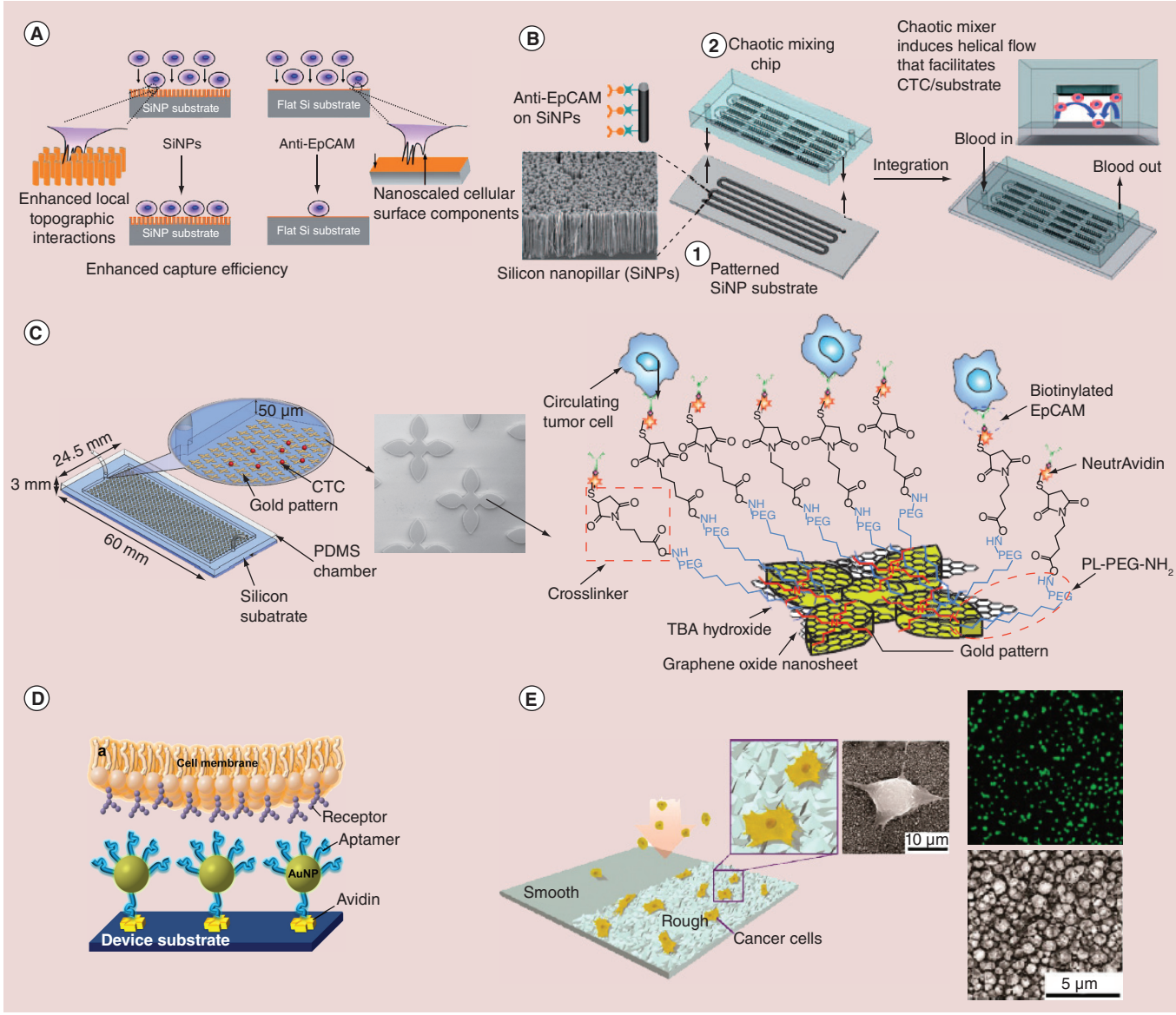

Figure 3. Nanostructured substrates for circulating tumor cells enrichment. **(A)** 3D silicon nanopillar substrates showing enhanced cell binding affinity as compared with the flat substrate. **(B)** Silicon nanopillar-embedded chaotic mixing microfluidic device. **(C)** Silicon substrates coated with graphene oxide nanosheet targeting CTCs through EpCAM recognition. **(D)** Gold nanoparticles-coated substrate to capture CTCs with aptamer ligands. **(E)** Roughened glass substrate showing capture of tumor cells without using CTC biomarkers.

CTC: Circulating tumor cell; PDMS: Polydimethylsiloxane; SiNP: Silicon nanopillar; TBA: Tetrabutylammonium.

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generation NanoVelcro devices can both capture (at 37°C) and release (at 4°C) over 90% tumor cells, with over 90% cells remaining viable.

Recently, Yoon *et al.* reported the use of graphene oxide (GO) to capture CTCs [74]. In their work, GO nanosheets were adsorbed onto a flower-shaped gold surface on a silicon substrate and chemically functionalized with EpCAM antibodies (Figure 3C). The recovery rates of 3–5 and 10–20 spiked breast cancer cells reached 73% and 94.2%, respectively, at flow rate of 1–3 ml/h. Without the GO sheet, the chip captures 13.3 and 48% for 3–5 and 10–20 cells, respectively. This demonstrated highly efficient CTC enrichment for low concentration CTCs.

For the substrates deposited with NPs (either by physical adsorption or by covalent binding), the multivalent effects play an important role in cell attachment. As demonstrated by Sheng *et al.* (Figure 3D), each gold (Au) NP (approximately 14 nm) is coated with 95 aptamer ligands [78]. When the Au NPs are coated on the channel of a microfluidic device, they enhanced cellular binding by 39-fold as compared with flat surface coated with aptamer alone. This increased the cell capture efficiency from 49 to 92%, indicating the strong promise of such nanostructured device for CTC capture and enrichment.

A marker- and size-independent methodology was lately reported by Chen *et al.* using nanoroughened substrates (Figure 3E) [80]. This method utilizes differential adhesion preference of cancer cells to nanorough surfaces when compared with normal blood cells. Using reactive ion etching, they made roughened glass surfaces with precisely controlled root-mean-square roughness from 1 to 150 nm. They found that as the roughness root-mean-square increases, capture yield of spiked cancer cells increases, reaching 80% for the 150-nm substrates. The device was tested with multiple breast cancer cell lines and compared with the flat surface that captures less than 20% cells in all tested cell lines.

CTC detection

Following isolation and enrichment, CTCs are detected and analyzed using either cytometric or nucleic acid-based approaches [83]. While cytometric methods analyze the cells based on protein expressions, the nucleic acid methods detect genetic alterations specific to tumor cells. Cytometric methods include immunohistochemistry imaging, spectroscopic detection and flow cytrometry. The advantage of cytometric methods over nucleic-acid based methods is the possibility to further characterize the cells since cell lysis is not required in the former procedures. When CTCs are examined microscopically, cell morphology can also been examined. Nucleic acid-based methods can analyze genetic information on whole cell or extracted RNA or DNA using PCR, RT-PCR, quantitative real-time RT-PCR, whole-genome amplification and FISH [84]. In general, nucleic acid methods have high sensitivity but low specificity due to interference from the expression of markers in normal cells. Nanomaterials are used in a variety of detection methods by taking advantages of their unique functional properties. Based on the mechanism of signal readout, the types of nanomaterials used in this area are classified as optical, magnetic and conductive NPs.

Optical nanoparticles

Fluorescent nanoparticles

Fluorescence is a leading technique for CTC detection and analysis. Generally, it is done with organic dyes as the imaging agents. However, it has been widely realized that the use of organic dyes is limited by photobleaching, low signal intensity, spectral overlapping and the need for multiple light sources to excited different fluorophores in multiplexed detection. Alternatively, quantum dots (QDs) have large absorption coefficient, narrow emission, high photostability and superior brightness [85]. Their emission can be precisely tuned by changing the size and composition of the NPs, which results in multicolor NPs with a single excitation laser source [86]. Due to these excellent properties, they have been widely used in biomedical imaging during the last decade [87]. However, they have not attracted much attention in CTC imaging [88–90]. The major concern is their cytotoxicity that may cause cell molecular changes and damage [91]. To avoid the toxicity issue, QDs can be used to detect CTCs by monitoring extracted nucleic acids. An example is the microfluidic bead-based nucleic acid sensor developed by Zhang *et al.* using multienzyme-nanoparticle amplification and QD labels [92]. By measuring the fluorescence signal intensity from QDs, the amount of targeted DNA can be quantified. The method can detect 1 spiked colorectal cancer cell in the blood.

Another class of fluorescent NPs is upconversion NPs (UCNPs) that contain lanthanide ions and show strong emission under NIR excitations [93]. Due to the NIR excitation, cellular autofluorescence is minimized and thus UCNPs enable imaging in biological samples with high sensitivity. In 2014, Fang *et al.* demonstrated the first application of UCNPs for CTC detection [94]. Using aptamer-linked NaYF4 (Yb:Er) UCNPs targeting PTK-7 on cancer cells, in combination with magnetic enrichment with superparamagnetic Av-conjguated Fe3O4 NPs, they showed linear correlation between the fluorescence intensity with the number of PTK-7 positive CCRF-CEM cells spiked in whole blood. As few as 10 cells spiked in 10 ml of whole blood were detected. The captured cells were further

examined with a confocal microscope, which indicated a purity of 70–90% depending on the concentration of spiked tumor cells.

Surface-enhanced Raman scattering nanoparticles

When organic dye with highly delocalized pi electrons is adsorbed onto metal NPs, the fluorescence signal of the dye is quenched and the Raman signals are strongly enhanced, as high as 1012–14 times leading to detection sensitivity down to single molecule and single particle level [95,96]. The dye-adsorbed metal NPs, termed surface-enhanced Raman scattering nanoparticles (SERS NPs), have emerged as a new generation of optical labels for biomedical imaging and diagnosis [97,98]. Different from the fluorescence technique, SERS gives sharp fingerprint-like signals (10–100-times narrower than fluorescence signals), distinct from biological autofluorescence/scattering background. This allows ultrasensitive detection without the need for tedious signal separation. The fingerprinting signals offer excellent multiplexity capability for multicolor imaging and detection [99,100]. The capability of using a single excitation source for multicolor probes and minimal photobleaching are additional advantages.

In 2008, Sha *et al.* demonstrated for the first time the potential of SERS NPs for CTC detection using spiked breast cancer cells [101]. 50 nm Au SERS NPs (Nanoplex Biotags) are linked with HER2 antibodies to target SK-BR-3 cells. By coupling with immunomagnetic enrichment with anti-EpCAM conjugated magnetic beads, the method can detect 10 tumor cells/ml blood. The studies used a sample holder with a magnetic assembly that focuses the tumor cells on a precise location on the wall of the tube for detection. A recent modified strategy was to magnetically enrich the labeled cells in a tube under a flow condition, followed by the detection by SERS technology [102]. The flow condition can be facilely translated into microfluidic modality for single cell analysis.

In 2011, Wang *et al.* developed a SERS-based assay using Au NPs covalently linked with epidermal growth factor peptide in combination with density gradient centrifugation enrichment and tested the assay with patient blood samples [103]. QSY21 quencher was used as the Raman reporter. The method can detect five to 50 spiked head and neck cancer cells in blood. Studies on clinic blood samples showed that CTCs in 17 out of 19 patients with head and neck cancer were detected, with CTC number ranging 1–720. A major advantage of this methodology is the specific detection of CTCs in the presence of WBCs, due to the use of small epidermal growth factor peptide rather than whole antibody ligands.

Recently, Zhang demonstrated the use of SERS NPs for CTC detection and enumeration at single cell level by combining a membrane substrate-based enrichment [104]. Nitrocellulose membrane substrate was functionalzed with anti-EpCAM antibodies to capture CTCs. The captured CTCs were labeled with Au SERS NPs linked with EpCAM antibodies. Microscopic SERS imaging was performed to detect and count CTCs. A significant advancement was made in the same year by Nima *et al.* who applied SERS for multicolor CTC imaging [105]. In their studies, four color SERS NPs were formed using Ag-coated Au nanorods (NRs) and four different Raman reporters (Figure 4A). Each color NP was linked with specific antibodies to target one of the four markers on breast cancer cells, IGF-1, anti-EpCAM, anti-CD 44 and anti-keratin 18. Using the SERS probe cocktail and the signature SERS peak from each reporter, the four markers on the same cell were imaged with a confocal Raman microscope. This represents a significant advancement in developing SERS-based technologies for CTC detection and analysis.

Magnetic nanoparticles

While MNPs have been typically utilized for CTC enrichment, they can be used as detection agents as well. In 2012, Weissleder, Lee and co-authors demonstrated an innovative technology using MNPs that can directly detect CTCs in whole blood and quantitatively measure specific biomarkers in a multiplexed fashion (Figure 4B) [106]. They developed a microfluidic chip-based micro-Hall detector (μHD) to detect induced magnetic moments by magnetically labeled cells in the presence of an external magnetic field based on the Hall effect. As the signal intensity is proportional to the number of bound MNPs and thus level of biomarkers, this method is able to detect and profile the targeted biomarkers on single cells in the presence of vast numbers of blood cells. Using MNPs of different sizes, they can profile multiple markers. The method has been tested with three different cancer cell lines and three different markers. The molecular profiles of these markers agreed well with those from flow cytometry. Studies on blood samples from 20 ovarian cancer patients showed that CTCs were detected in 100% patients in contrast to 20% patients with the CellSearch system. The method detected a higher number of CTCs in patients of ovarian cancer than CellSearch across all the patient samples due to the use of a multiplexed targeting strategy. A potential drawback with this technology is that CTCs with low level of protein expressions may not be detected because the tumor cells require over 106 MNPs in order to be detected.

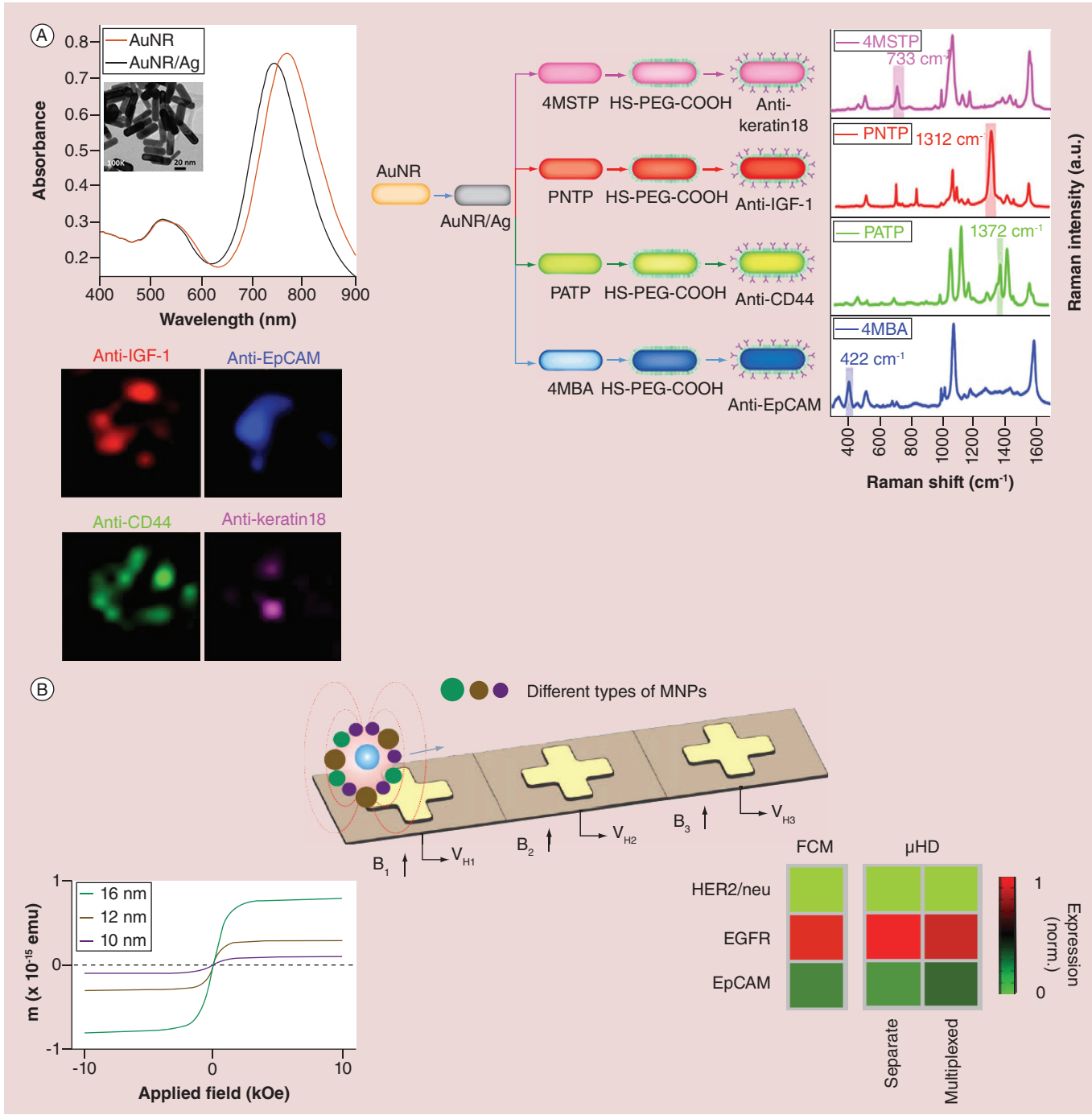

Figure 4. Nanoparticles for multiplexed detection, imaging and profiling of circulating tumor cells. **(A)** Multiplexed imaging of circulating tumor cells using four color Au-Ag core-shell SERS nanorods. Top Left: Absorption and TEM image of Ag-Au core-shell nanorods. Red: Au nanorods. Black: Au-Ag core-shell nanorods. Right: Preparation and SERS spectra of 4 color Au-Au SERS nanoprobes. Down left: Raman imaging of tumor cells with four color Au-Au SERS nanoprobes. **(B)** Multiplexed detection and profiling of CTCs with $MnFe_2O_4$ MNPs in combination with a micro-Hall detector. Top: Schematic showing the principle of multiplexed profiling of CTC marker expressions using MNPs of different sizes in conjunction with a micro-Hall detector. Down left: Magnetic properties of MNPs with different sizes. Down right: The heat map showing comparison of the relative expression levels of three different tumor makers measured using the micro-Hall detection method and flow cytometry.
MNP: Magnetic nanoparticle; MSTP: 4-(methylsulfanyl) thiophenol; PNTP: P-nitrobenzoic acid; PATP: P-aminobenzoic acid; 4MBA: 4-mercaptobenzoic acid.
(A) Reprinted with permission from [105] © Macmillan Publishers Ltd: (Science Reports; 2014); **(B)** Adapted with permission from [106] and reprinted with permission from © AAAS (2012).
For color images please see online at: www.futuremedicine.com/doi/full/10.2217/NNM.15.32

With a different detection mechanism, the same groups developed a micronuclear magnetic resonance (μNMR)-based method for detecting and counting CTCs in whole blood [107]. The μNMR measures the transverse relaxation time (T2) of water proton in a solution sample. When the sample contains CTCs labeled with MNPs, the MNPs produce local dipole fields with strong spatial dependence, which accelerates the transverse relaxation of water protons and thus shorter T2 than nontargeted objects [108]. The method involves RBC lysis after MNP labeling. Experiments with spiked cancer cells showed recovery rates of 30–45% depending on the concentration of the tumor cells. The method detected CTCs in 87% of patients with ovarian cancer, with higher CTC number than the CellSearch system in advanced cases.

Conductive nanoparticles

Carbon nanotubes (CNTs) have remarkable electronic properties, behaving as a metal or semiconductor depending on their diameter and helicity [109]. Mechanical deformations or chemical binding can induce strong variations of its conductance, which can be easily detected by electron current signals. Such unique properties make CNTs excellent chemical and biological sensors. In 2008, Shao *et al.* demonstrated the first application of CNTs for electric detection of cancer cells in blood. They made single wall CNT field effect transistor array device containing 20 pairs of electrodes with a single CNT between each pair. The CNTs were functionalized with antibodies to recognize breast cancer cells. The binding of cancer cells to the CNTs induced 60% decrease in conductivity whereas control experiments produced less than 5% decrease in conductivity. The key advantage of this assay is that the sensing area is limited to few receptors in cells and thus may potentially detecting CTCs with low protein expressions. It also directly detects cancer cells in blood, without the need of preenrichment. However, it has difficulty to count CTCs because the signal is only determined by a single cell reaching the spacing between the electrodes. The volume of analyzed blood is also very small (<10 μl), which may miss rare CTCs in patient blood.

A recent study by Liu *et al.* demonstrated a quantitative CNT-based sensor for direct detection of cancer cells in whole blood using real time electrical impedance sensing [110]. Multilayer CNTs were assembled on an indium tin oxide electrode surface and modified with EpCAM antibodies. Cell binding induced increase of the electron-transfer resistance. Using liver cancer as a model, they demonstrated that this electrical response was linearly proportional to the concentration of the cancer cells in whole blood, with a detection of limit of five cells per ml of blood. Rather than whole cell detection, Kwon described a method to detect proteins collected from lysed cancer cells using CNT patterned surface coupled with scanning probe microscopy imaging [111]. CNTs served as a substrate to recognize carcinoembryonic antigens (CEAs) expressed on CTCs and scanning probe microscopy was used to image CEAs bound on the CNTs. This quantitative assay can detect not only a single CTC but also single CEA molecule, indicating its superior sensitivity.

Dual enrichment & detection with hybrid nanoparticles

Due to their scarcity of CTCs in blood, materials that allow dual enrichment/detection modalities are highly desirable for developing simple, rapid and efficient detection methods. The dual functional materials bridge the gap between the enrichment and detection technologies, simplifying sample processes and minimizing CTC loss and damage. Thus, dual functional nanomaterials are very promising for developing new generation of CTC detection technologies.

An earlier strategy was developed by Maeda *et al.* who made magnetic-fluorescent nanocomposites with bacterial MNPs (BacMPs) and commercial QDs for dual magnetic isolation and fluorescence detection [112]. The BacMPs were functionalized with anti-EpCAM using gene fusion techniques. In their proof-of-concept studies, the nanocomposite, under optimized conditions, led to a 90% recovery rate for NCI-H358 lung cancer cells from 1 ml of PBS suspension whereas less than 10% for JM non-epithelial model cells. Following magnetic separation, the cancer cells were detected by fluorescence imaging because of the QD moieties in the composites. Magnetic-fluorescent composites using MNPs and organic dyes have also been reported [113,114]. Recently, Ray and co-authors used Fe3O4-Au core-shell NPs and organic dye to form the hybrid nanosystem [115]. The use of magnetic-plasmonic core-shell NPs allows subsequent destruction of captured tumor cells with photothermal therapy.

We recently developed a dual capture and detection assay using highly integrated magnetic-plasmonic core-shell SERS NPs (Figure 5) [116]. We synthesized Fe3O4-Au core-shell NPs in different shapes based on a seed-mediated growth method [116,117]. These anisotropic core-shell NPs are 30–40-times higher in SERS activities than traditional spherical counterparts. The integrated nanoprobes were made from oval-shaped Fe3O4-Au core-shell NPs, which were adsorbed with QSY21 Raman reporters and linked with one of two different antibodies, EpCAM or HER2. To further minimize cell loss, we constructed an online cap-

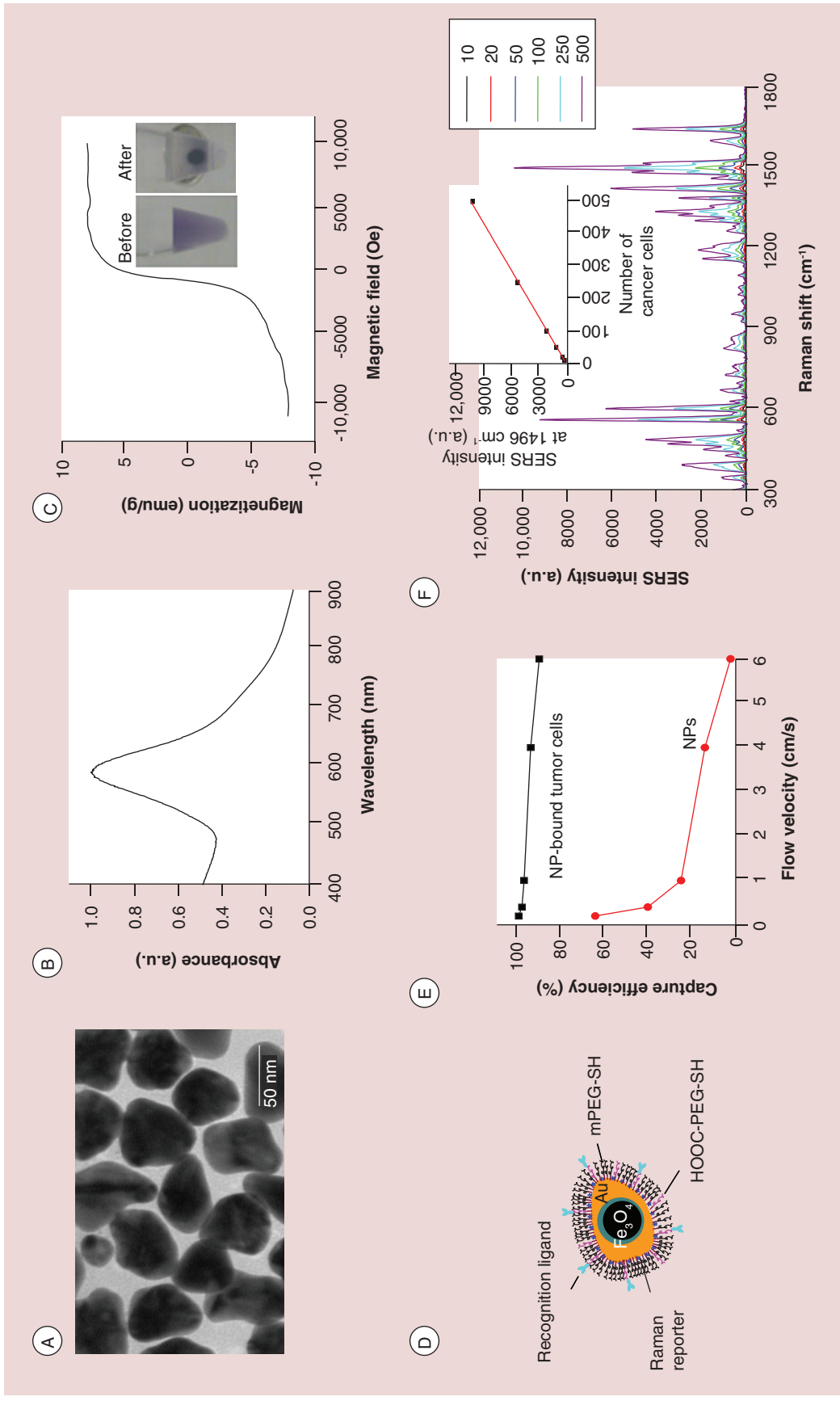

Figure 5. Magnetic-plasmonic hybrid nanoparticles for dual enrichment and detection of circulating tumor cells. **(A)** TEM image, **(B)** absorption spectrum and **(C)** M-H curve and magnetic separation micrograph of oval shape IO-Au core-shell NPs. **(D)** Structure of oval shape IO-Au SERS nanoprobes. **(E)** Capture efficiencies of nanoparticle-bound SK-BR-3 tumor cells (black) and free oval shale IO-Au core-shell nanoparticles (red). **(F)** Detection of spiked SK-BR-3 cells in whole blood with oval shape IO-Au SERS nanoprobes in combination with an on-line magnetic enrichment and SERS detection system. The limit of detection is calculated to be 1-2 cells/ml blood.
NP: Nanoparticle.
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ture and detection system, in which tumor cells were firstly captured online with a macromagnet and then immobilized by a micromagnet in the absence of the macromagnet for SERS detection. Using the duplex targeted nanoprobes in combination with the online flow system, we demonstrated that over 90% SK-BR-3 breast cancer cells can be captured at a flow velocity of 6 cm/s without significant interference from free NPs. Spiking experiments with SK-BR-3 cells showed that the intensity of the SERS signals from the Raman reporter was linearly correlated to the number of cells in the whole blood. Our method showed a limit of detection of 1–2 cells per milliliter of human whole blood. With further development using microfluidic device, multiplexed targeting and microscopic Raman detection, the technique will lead to a new generation of versatile system for highly sensitive and specific detection and profiling of CTCs in whole blood.

In vivo enrichment & detection with nanomaterials

Techniques capable of detecting and quantifying CTCs *in vivo* are valuable because they are noninvasive and can monitor CTC level in real-time. They detect CTCs in the entire blood volume of the body, which can enhance sensitivity up to 102–103 times

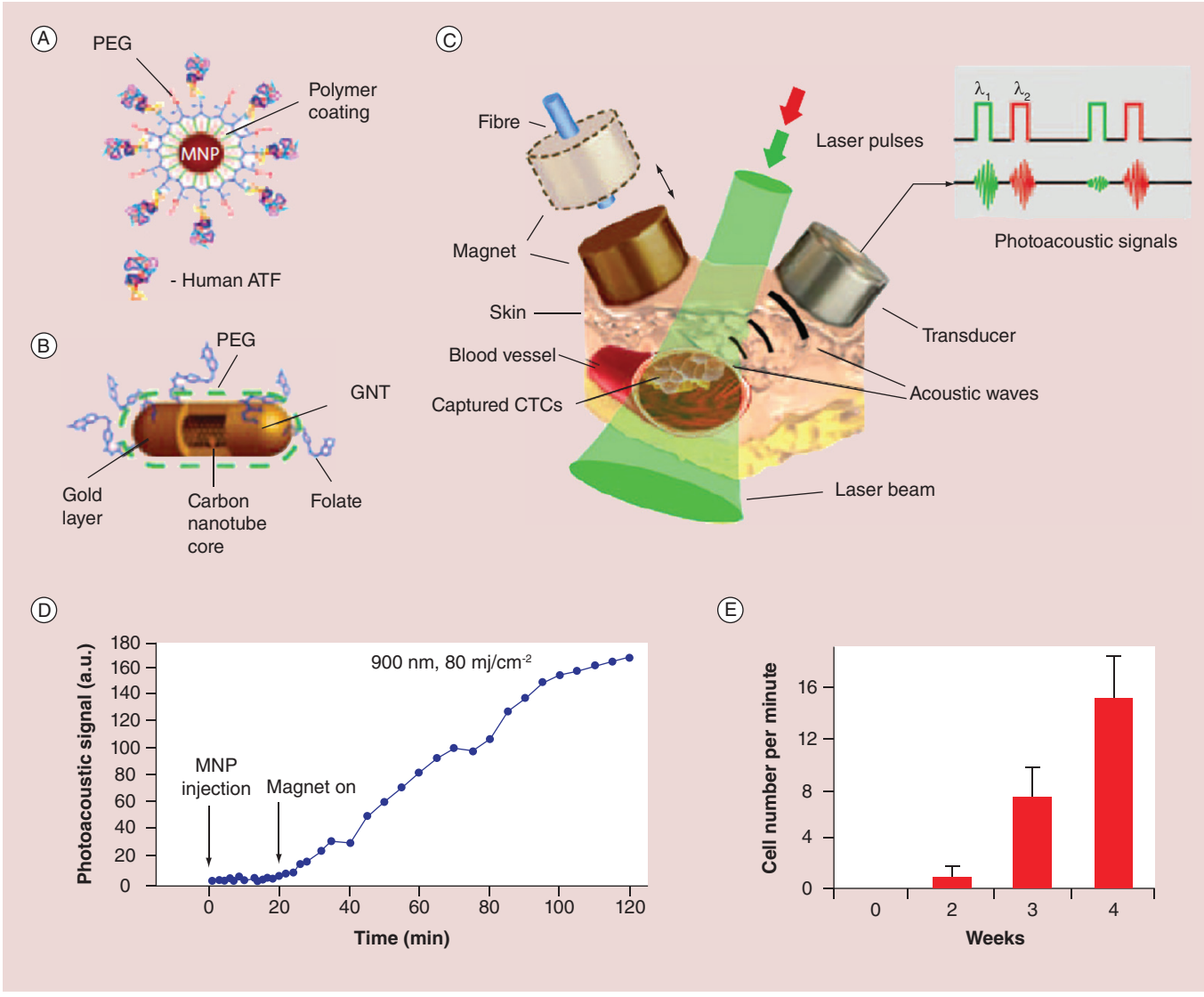

Figure 6. Nanoparticles for *in vivo* magnetic enrichment and photoacoustic detection of circulating tumor cells. (A) MNP probes targeting urokinase plasminogen activator receptors on tumor cells. **(B)** GNT probes targeting folate receptors on tumor cells. **(C)** Enrichment and detection setup. **(D)** Photoacoustic signals from CTCs in abdominal vessels at week 1 of tumor development with and without magnetic enrichment. **(E)** The average CTC rate in mouse ear vein over a period of 4 weeks after tumor development. ATF: Aminoterminal fragment; CTC: Circulating tumor cell; GNT: Gold nanotube; MNP: Magnetic nanoparticle; Reprinted with permission from [130] © Macmillan Publishers Ltd (2009).

as compared with *ex vivo* methods [118]. *In vivo* CTC detection has been done on superficial vessels with a mouse model by *in vivo* flow cytometry using traditional fluorescence [119–126] or newly developed photothermal (PT) and photoacoustic (PA) [127–134] detection methods, or a combination of these methods [135,136].

An example of the use of nanotechnology in CTC detection with *in vivo* fluorescence flow cytometry was the use of two color QDs (Qdot 585 and Qdot655) to track the circulation of breast cancer cells of two different cell lines [122]. This work, reported by Tkaczyk *et al.*, was the first study showing that two different populations of circulating tumor cells can be quantified simultaneously in the blood circulation in a mouse model. The technique has the potential to track different CTC subtypes *in vivo*. However, limitations of the fluorescence-based methods exist, including potential cytotoxiciy of QDs and interference from light scattering and autofluorescence. These drawbacks can be avoided in PT/PA detection methods, which are based on the absorption properties of light from pulsed laser by endogenous biomolecules or exogenous contrast agents and subsequent nonradiative relaxation of absorbed laser energy into heat. In PT imaging, the heat induces the variations of the refractive index in the cells, which is detected with phase-contrast imaging technique with a second, collinear probe laser pulse [137]. In PA flow cytometry the temperature fluctuations resulting from the pulsed heating by the laser generates pressure waves that is detected with an ultrasound transducer [138]. PAFC combines high sensitivity and spectral specificity of optical methods with high spatial resolution and tissue penetration of ultrasounds methods, and thus very promising for *in vivo* detection.

Using low toxicity metal-based NPs, Zharov and co-authors have conducted extensive studies on CTC detection with *in vivo* PT/PA flow cytometry from single color to two color detection [130–133]. In their studies, a groundbreaking approach is the use of MNPs and gold coated carbon nanotube for magnetic enrichment and PA detection of CTCs *in vivo* (Figure 6) [130]. In this method, 10 nm Fe2O3 NPs were functionalized with the aminoterminal fragment (ATF) of the urokinase plasminogen activator to target urokinase plasminogen activator receptor on breast cancer cells. The MNPs served as dual magnetic and PA contrast agents. GNTs have a higher PA contrast than MNPs in the NIR region and thus were used as a second PA agent to increase detection sensitivity. GNTs were also used as a second targeting agent through linked folate ligands to increase detection specificity. Results showed that the sensitivity limit is 35 GNTs and 720 MNPs. Selective capture (over 90%) of MNP-labeled cancer cells over free MNPs were achieved at a flow velocity of 2–8 cm/s. The technique was examined by detecting CTCs in the blood circulation of a nude mouse with breast cancer xenografts through the ear vein. After injection of a cocktail of the two nanoprobes, they observed photoacoustic signals when the magnet is placed near the ear vein. The signals further increased with time, indicating successful enrichment of CTCs. By converting the signals into cell number, they were able to monitor CTC level during tumor development. They observed that the CTC rate increased dramatically with time, correlating to different stages of tumor. Zharov and co-authors further demonstrated the ability to kill CTCs *in vivo* following detection using photothermal melanin NPs [129] or cancer stem cells (CSCs) using GNTs [131]. Incorporation the killing modality with the detection methods is beneficial because it directly reduces the risk of metastasis by eradicating these diseased cells.

Conclusion

Nanotechnology receives intense attention for the enrichment and detection of CTCs during the last decade owing to the unique structural and functional properties of nanoscale materials. Magnetic NPs are used for the enrichment of CTCs in blood samples in the FDA-approved CellSearch system. Many new nanotechnology-based techniques that improve upon the CellSearch system have been developed and showed strong potential for clinical applications. Most techniques use NPs, such as magnetic, fluorescent, metallic, conductive NPs or nanostructured substrates, to enrich or detect CTCs in blood samples. Hybrid NPs have been recently developed to bridge current enrichment and detect methodologies. Nanotechnology has also been used to detect CTCs *in vivo*. The use of photothermal NPs in the *in vivo* technologies allows subsequent eradication of CTCs, which is promising for prevention of cancer metastasis.

Future perspective

In the next 5–10 years, sensitivity and specificity remain the key issues to be addressed in future technologies. The dual functional nanomaterials such as magnetic-optical core-shell nanoparticles are very promising to reduce false negatives by bridging current enrichment and detection methods. Multiplexed targeting is another way to improve detection sensitivity by capturing and analyzing CTC subpopulations. Nanomaterials are ideal for developing multiplexed assay as each particle can be linked with different targeting ligands. In addition, incorporation of nanomaterials with microfluidic devices will continue to be an optimistic strategy in CTC enrichment and detection because of the combined benefits of nanotechnology and microchip technology.

Another future direction of the field will be molecular characterization of CTCs that may better inform clinical decision making. The ability to reliably and efficiently characterize CTC gene and protein expression profiles remains limited. The SERS NPs may revolutionize protein analysis at the single cell level. SERS NPs can profile CTC protein markers in a high throughput fashion. Multicolor SERS NPs (>10 colors) can be formulated without changing the size and shape of the nanostructures. Signals from each color NPs can be facilely obtained by deconvolution with classic least square regression. Thus, the multicolor SERS NPs will lead to novel characterization methods in a high throughput fashion.

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Acknowledgements

The authors thank the financial support by NIH (Grant No: 1R15 CA 195509-01) and the University of Memphis FedEx Institute of Technology Innovation Fund.

Financial & competing interest disclosure

The authors acknowledge the NIH funding ageny (Grant No 1R15CA195509-01). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Background

- Circulating tumor cells (CTCs) provide a potentially noninvasive liquid biopsy for characterizing and monitoring cancer, helping inform clinical decision making.
- A variety of techniques have been developed to enrich and detect CTCs during the last two decades, with the CellSearch system being approved by FDA for utilization in the clinic to count CTCs in blood of patients with metastatic breast, prostate and colon cancer.
- Nanotechnology plays an important role in CTC enrichment and detection with increasing interests in recent years owing to the unique structural and functional properties of nanoscale materials.

Nanotechnology in enrichment & detection of CTCs

- Magnetic nanoparticles have been widely used for CTC enrichment based on classic bulk immunomagnetic separation or microchip-based immunomagnetic separation.
- A variety of nanostructured substrates have been developed to capture and enrich CTCs.
- Magnetic, fluorescent, plasmonic and conductive nanoparticles have been recently used to detect CTCs either directly in whole blood or in combination with enrichment methods.
- Hybrid functional nanoparticles have emerged as new contrast agents for dual enrichment and detection of CTCs.
- *In vivo* enrichment and detection technologies have been developed based on magnetic and plasmonic nanoparticles.

Conclusion

- Nanomaterials are used in the US FDA-approved CellSearch system.
- Many new technologies with significant improvement and great potential for clinical applications have been developed using various nanoplatforms, mainly magnetic, fluorescent, metallic and conductive NPs as well as nanostructured substrates.
- Nanotechnology has been proven promising for *in vivo* detection of CTCs.

Future perspective

- Sensitivity and specificity remain the key issues to be addressed in future technologies.
- Dual functional nanoparticles with multiplexing capability are very promising in developing new generation of CTC enrichment and detection technologies.
- Nanomaterials are very promising for molecular characterization of CTCs, especially for profiling of CTC protein expressions at the single cell level.

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