Chapter 3
Aptamer-Based Methods for Detection of Circulating Tumor Cells and Their Potential for Personalized Diagnostics

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Abstract Cancer diagnostics and treatment monitoring rely on sensing and counting of rare cells such as cancer circulating tumor cells (CTCs) in blood. Many analytical techniques have been developed to reliably detect and quantify CTCs using unique physical shape and size of tumor cells and/or distinctive patterns of cell surface biomarkers. Main problems of CTC bioanalysis are in the small number of cells that are present in the circulation and heterogeneity of CTCs. In this chapter, we describe recent progress towards the selection and application of synthetic DNA or RNA aptamers to capture and detect CTCs in blood. Antibody-based approaches for cell isolation and purification are limited because of an antibody's negative effect on cell viability and purity. Aptamers transform cell isolation technology, because they bind and release cells on-demand. The unique feature of anti-CTC aptamers is that the aptamers are selected for cell surface biomarkers in their native state, and conformation without previous knowledge of their biomarkers. Once aptamers are produced, they can be used to identify CTC biomarkers using mass spectrometry. The biomarkers and corresponding aptamers can be exploited to improve cancer diagnostics and therapies.

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3.1 Introduction

Oncological diseases are the second leading cause of death after cardiovascular diseases. Early clinical diagnosis of pre-metastatic malignancy tumors could increase treatment efficiency and survival rate of cancer patients. One of promising tools for better lung cancer diagnosis and monitoring during treatment is evaluation and enumeration of circulating tumor cells (CTC) in blood (Punnoose et al. 2012). There are numerous methods for isolation and concentration of rare cancerous cells from blood, such as dielectrophoresis and stepping electric fields (Hatanaka et al. 2011; Chen et al. 2014), fluorescence-activated cell sorting (FACS), magnetic-activated cell sorting (Jacob et al. 2007). In general the methods for enriching CTCs can be divided into two categories: size-based and immunomagnetic approaches, the advantages and disadvantages of which have been addressed in the literature (Alunni-Fabbroni and Sandri 2010). Among immunomagnetic methods, the CellSearch system has been approved for clinical use by the U.S. FDA (Allard et al. 2004). It relies on antibody-based capture and staining of epithelial cell adhesion molecule (EpCAM) as well as Cytokeratins 8, 18, or 19.

Unfortunately, no single antibody is sufficient to capture and detect all rare CTCs. For instance, EpCAM is not a perfect marker for CTC detection due to the high variation in its gene expression between tumor subtypes (Lampignano et al. 2016). Many CTCs also express epithelial, mesenchymal, and stem-cell markers.

The CTC research has sparked considerable interest in the application of molecules that offer similar or enhanced functionalities to antibodies, but that can be easily synthesized with additional characteristics. Aptamers have enormous potential as such molecules. These small (5–30 kDa), single-stranded DNA and RNA molecules carry the blueprint for their own synthesis in their primary sequence, so they can be synthesized by pure chemical procedures. They fold into well-defined three-dimensional structures and show high affinity and specificity for their targets. In many respects, aptamers are superior to antibodies. They can be selected through an *in vitro* evolution process using live cancer cells or primary tumors in a few days without knowing beforehand cell specific biomarkers. The resulting aptamers are chemically synthesized in high purity at low cost (1000s times cheaper than the production of monoclonal antibodies), and are considered to be a synthetic chemical product, rather than a biological product. For isolation purposes aptamers can be easily removed from target cells washing out with chelators or nucleases (Jayasena 1999; Li et al. 2013; Shen et al. 2013; Wehbe et al. 2015) or DNA complementary sequences (Rusconi et al. 2002). In contrast antibody releasing from its targets requires harmful to cells harsh chemical or enzymatic treatments (Sheng et al. 2014).

3.2 Microfluidic Aptamer-Based Devices for CTC Capture

Currently, aptamer-based technologies are widely studied in various biomedical fields. Over 6000 published articles on aptamers are referenced in the PubMed database, and more than 1000 of them are related to cancer. The first microfluidic device utilizing the aptamer to EGFR for isolating cancer cells from a laminar flow of human mononuclear cells has been reported by Wan et al. (2011). Two following microfluidic devices have been shown in 2012. The first device utilized elliptical (30 μm × 15 μm) micropillars 32 μm in height and an interpillar distance of 80 μm in flow channels (Fig. 3.1) (Sheng et al. 2012). The micropillars were coated with DNA aptamers previously selected against the following cancer cell lines: CCRF-CEM cells, Ramos cells, HCT 116 cells, and DLD-1 cells (Martin et al. 2011; Sefah et al. 2010; Shangguan et al. 2006; Tang et al. 2007). Less than 30 min was enough to isolate as few as 10 colorectal tumor cells from 1 ml of unprocessed whole blood using this small device. The cancer cells captured with the micropillar flow chamber were viable and used for following cellular and molecular characterization.

The second Hele-Shaw microfluidic device with plain surface of the channel and with array of pits on the channel floor have been utilized for the efficient detection of rare cancerous cells (Fig. 3.2). The pits were filled with anti-EGFR aptamer functionalized glass beads. Cancer cells, flowing in solution through the channel, were captured by the beads with high specificity. Such design helped sorting cell sub-populations with varying EGFR expression. Cells were released from the beads by a complementary to oligonucleotide sequence. This approach isolated viable cells for further analysis (Wan et al. 2012).

A flat channel microfluidic device made of polydimethylsiloxane and functionalized with locked nucleic acid (LNA) aptamers targeting EpCAM and nucleolin has been developed by Maremanda et al. for quick and efficient capture of CTCs and cancer cells. The analysis of blood samples obtained from 25 head and neck cancer patients detected as small as 5 ± 3 CTCs in ml of blood. These microfluidic devices also maintained viability for *in vitro* culture and characterization (Maremanda et al. 2015).

Recently, another microfluidic assay has been developed using a cocktail of aptamers with a synergistic effect. When a single aptamer was employed in the chip composed of silicon nanowires and an overlaid PDMS chaotic mixer, the capture affinity of the device was relatively weak. Nevertheless, using several aptamers, the synergistic effects among individual aptamers lead to an enhanced capture affinity (Fig. 3.3). It has been shown that for the patients with nonsmall cell lung cancer (NSCLC) this method provided more comprehensive information in treatment monitoring (Zhao et al. 2016).

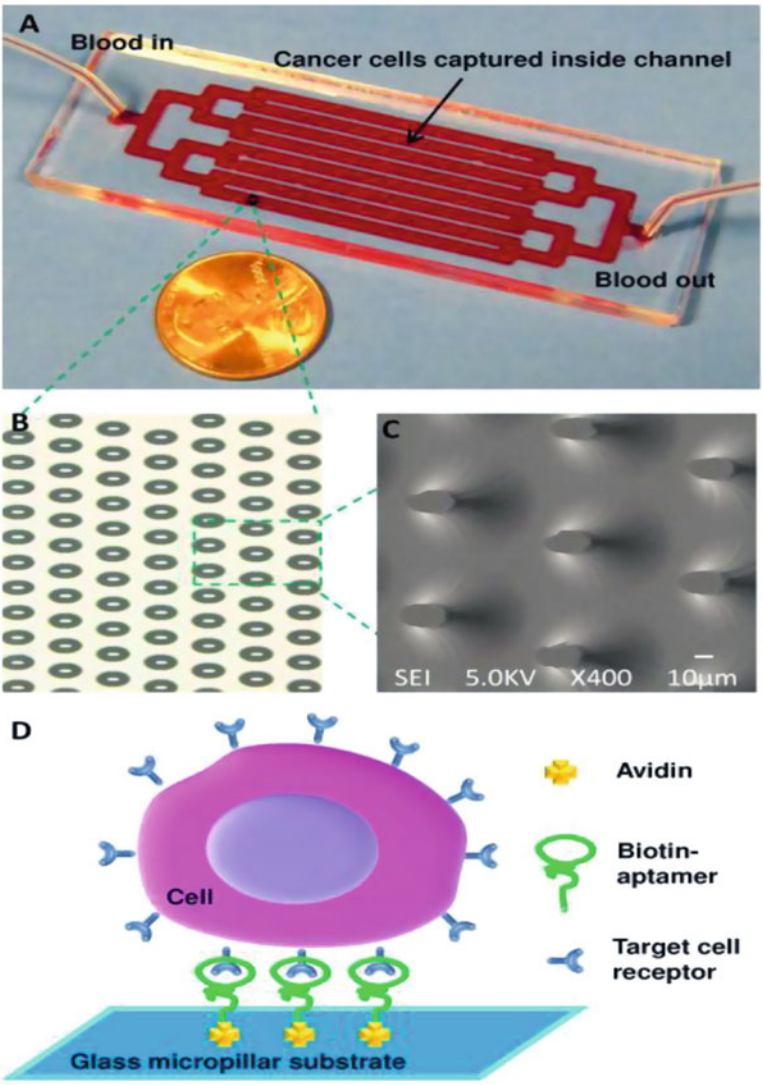

Fig. 3.1 Microfluidic micropillar device for cancer cell isolation (**a**) Optical micrograph (**b**) and scanning electron microscope images of a micropillar array in a channel in the glass substrate (**c**) Scanning electron microscopy image of elliptical micropillars (**d**) Scheme of capturing cancer cells in the device (From Sheng et al. 2012, with permission)

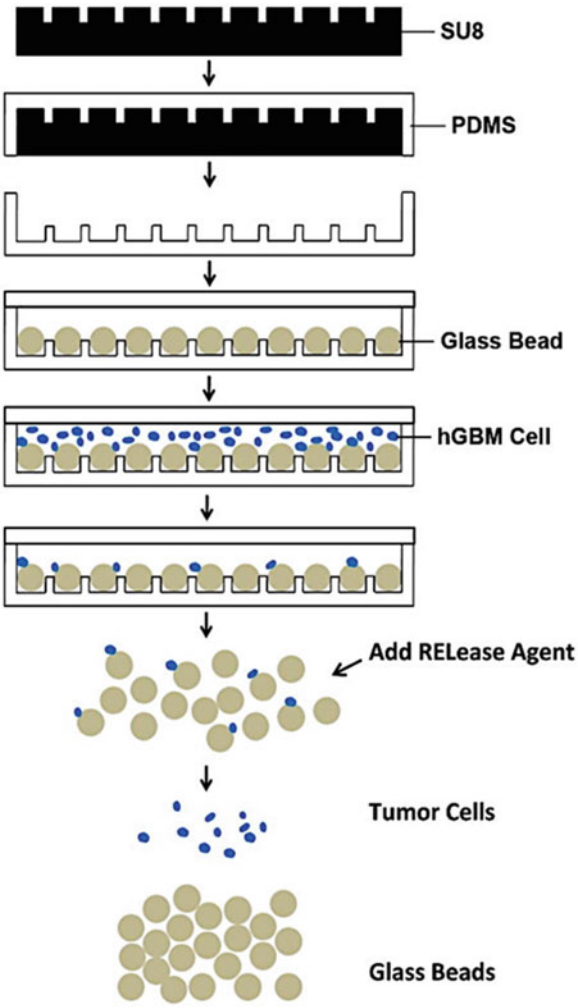

Fig. 3.2 Scheme of fabrication and application of Hele-Shaw microfluidic device. SU-8 photoresist is spin-cast on silicon wafer, exposed and wells are developed to form the desired pattern. PDMS is poured on SU-8 master, baked, and peeled off. 50 μm diameter glass beads (GBs) are loaded into 25 μm deep pits and the substrate is covered with a flat PDMS slab. Cancer cell suspension is flowed through the device, and cells are captured by aptamers-functionalized GBs. Captured cells are finally released from the GB surface after GBs are collected from the device (From Wan et al. 2012, with permission)

3.3 CTC Isolation with Aptamer-Functionalized Nanoparticles

Chitosan nanoparticle surface fabricated by electrospray was modified by polyethylene glycol and a DNA aptamer for a specific capture of viable rare CTCs from artificial white blood cell samples. Furthermore, an *in situ* culture strategy has been proposed (Fig. 3.4). This work provides a promising strategy for viable isolation and purification of rare CTCs and it has great potential for achieving clinical validity (Sun et al. 2015).

Aptamer-functionalized gold nanoparticles could be used to enrich and detect cancer cells using an aptamer-nanoparticle strip biosensor within a lateral flow

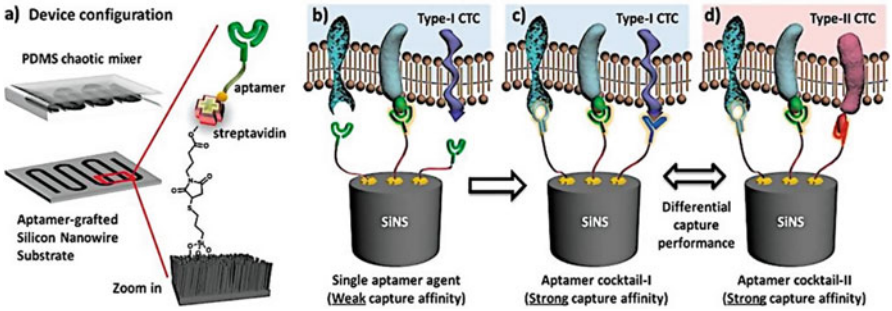

Fig. 3.3 Scheme of an aptamer cocktail based CTC assay. Microfluidic CTC chip is composed of an aptamer-grafted silicon nanowire substrate and an overlaid PDMS chaotic mixer (**a**) When a single aptamer capture agent was employed, the capture affinity of the device is relatively weak for the lack of synergistic binding (**b**) By using cocktail capture agents, the synergistic effects among individual aptamers lead to an enhanced capture affinity (**c**) Different cocktail capture agents are expected to have differential capture performance for CTC subpopulation recognition (From Zhao et al. 2016, with permission)

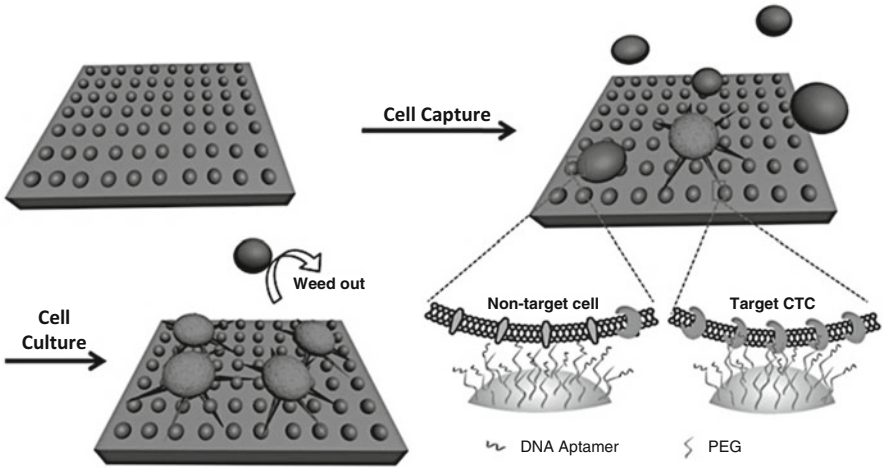

Fig. 3.4 Scheme of the chitosan nanoparticle substrate for rare number CTC isolation from non-target cells followed by the optimized in situ culture of captured cells (From Sun et al. 2015, with permission)

device (Fig. 3.5) (Liu et al. 2009). A pair of aptamers to Ramos cells – a thiolated aptamer (TD05) immobilized on gold nanoparticles and a biotinylated aptamer (TE02) immobilized in the test zone of a nitrocellulose membrane. The lateral flow strip device allowed cancer cells linked through TD05 aptamer with gold nanoparticles stay in the test zone. Accumulation of colloidal gold produced a visible red band. Unfortunately the large volumes of blood (more than 5 μL) masked the signal from the cancer cells because of the non-specific adsorption of

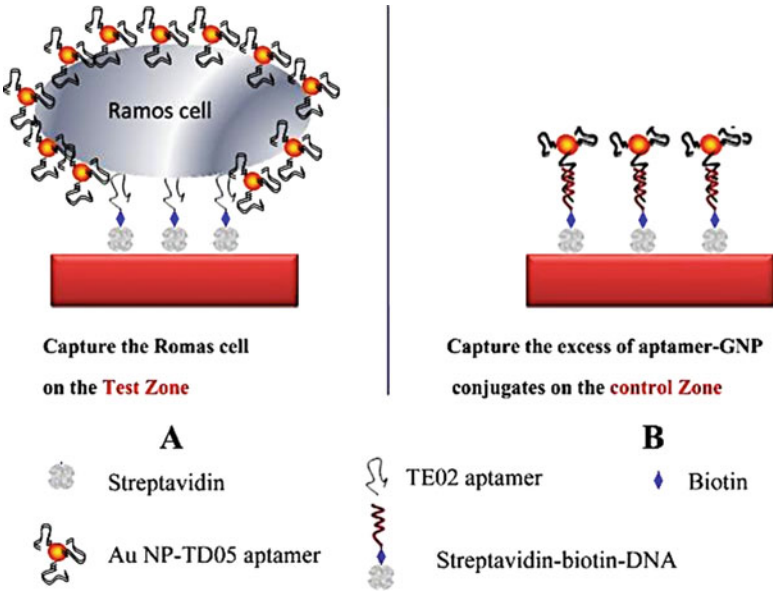

Fig. 3.5 Scheme of the detection of Ramos cells on aptamer-nanoparticle strip biosensor (**a**) Capturing Au-NP-aptamer-Ramos cells on the test zone through specific aptamer-cell interactions (**b**) Capturing the excess of Au-NP-aptamer on the control zone through aptamer-DNA hybridization reaction (From Liu et al. 2009, with permission)

erythrocytes on the membrane. The further optimization of this technology is required for the clinical implementation (Liu et al. 2009).

Technologies combining aptamer-functionalized nanoparticles with microfluidics can greatly enhance the robustness of aptamer-based CTC capture. A laminar flow flat channel microfluidic device allowed capture CCRF-CEM cells from blood due to the aptamer Sgc8 immobilized on gold nanoparticles (Sheng et al. 2013). The problem of the cancer cell diversity could be solved by using aptamer-functionalized barcode particles to capture and detect various types of CTCs (Fig. 3.6). Variations in reflection properties of different spherical colloidal crystal clusters each modified with an aptamer to a certain CTC type, act as a code for analyses. Dendrimers were used to amplify the effect of the aptamers, allowing for increased sensitivity, reliability, and specificity in CTC capture, detection and release (Zheng et al. 2014).

3.4 Electrochemical Aptasensors for CTC Detection

Another promising approach for potential CTC-related clinical applications is rather simple and ultrasensitive electrochemical sensor based on the cell-specific aptamer-modified glassy carbon electrode (GCE) detecting as few as a single BNL

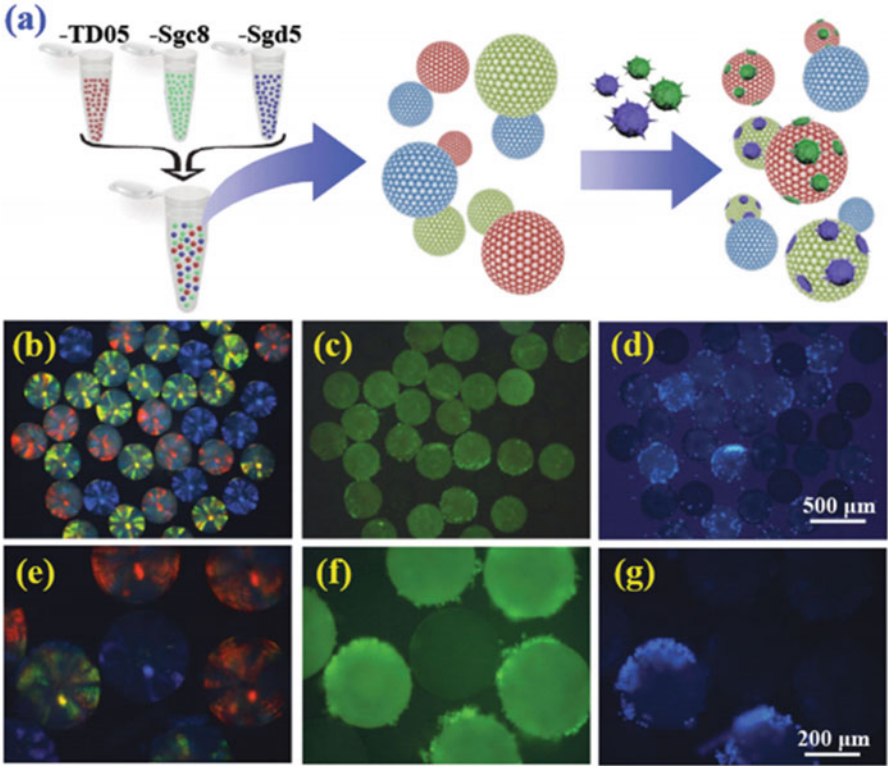

Fig. 3.6 Scheme showing the barcode particles capturing multiple types of CTCs. Various aptamers, TD05, Sgc8, and Sgd5, were used; and *green* and *blue*-stained cells were used as the target cells (From Zheng et al. 2014, with permission)

1ME A.7R.1 (MEAR) mouse hepatoma cell in 10^9 whole blood cells (Fig. 3.7) (Qu et al. 2014).

An RNA-aptamer biochip was developed for capturing and detecting a single tumor cell. The polydimethylsiloxane chip consisted of a cover containing a channel for introducing cells and sustaining their activity and microelectrode matrix on a silicon dioxide layer. The anti-EGFR RNA aptamers specifically bound the tumor cells, allowing the detection of a single cell due to the increase of ion current resistance between electrodes. This novel approach demonstrated the isolation of CTCs from peripheral blood, counting and follow-up gene or protein analysis (Wang et al. 2012).

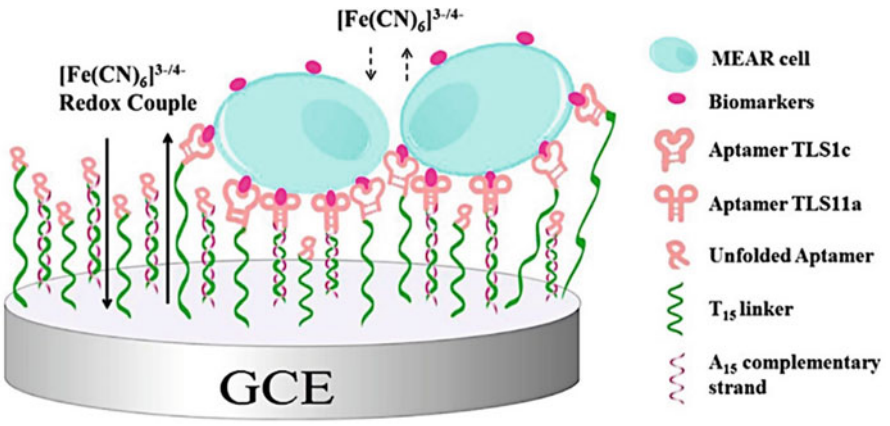

Fig. 3.7 Scheme of a dual modified electrode for specific and sensitive detection of tumor cells. Two MEAR cell-specific aptamers, TLS11a and TLS1c, conjugated to the surface of a glassy carbon electrode (GCE) via a rigid dsDNA linker (T15/A15) and a flexible ssDNA linker (T15), respectively. Specific binding brings a dramatic steric hindrance effect on the electron transfer of the redox couple $[Fe(CN)_6]^{3-/4-}$ through the GCE, while the electrostatic repulsion between negative charges of the cell surface and $[Fe(CN)_6]^{3-/4-}$ may further inhibit the electron transfer, thus significantly reducing the electron transfer speed (From Qu et al. 2014, with permission)

3.5 Aptamers for Fluorescent CTC Detection

One-step fluorochrome-quencher based strategy was described by Zeng et al. (2014) on the basis of an anti-CD30 RNA aptamer. In the absence of cells it did not emit fluorescence (Fig. 3.8), but when this aptamer-based probe interacted with a target cell, it was internalized and trafficked to the lysosome and where it was degraded. The quencher was separated from the fluorochrome, thereby allowing it to emit fluorescence. This was successfully used to identify CTCs in the whole blood of lymphoma patients, with a little background signal from the blood cells.

Another method for highly efficient capture and accurate identification of multiple types of blood CTCs using aptamer-modified porous graphene oxide membranes has been announced in 2015 by Ray group (Fig. 3.9). Aptamers to different cell types attached to 20–40 μm porous garphene oxide membranes were capable of capturing multiple types of tumor cells (SKBR3 breast cancer cells, LNCaP prostate cancer cells, and SW-948 colon cancer cells). The efficiency of graphene oxide membranes was about 95% for multiple types of tumor cells. Each aptamer had a different fluorescent dye conjugated at the 5′ end for multicolour fluorescence imaging (Nellore et al. 2015).

Another work opens up the possibility for personalized diagnostics, demonstrating advantages of using the aptamers over the antibodies, by allowing the detection of heterogenic biomarkers of tumor tissues from the individual patient. A novel in situ tissue slide-based SELEX strategy has been developed by Zhang et al. (2015). DNA aptamers that bind to formalin-fixed, paraffin-embedded breast infiltrating

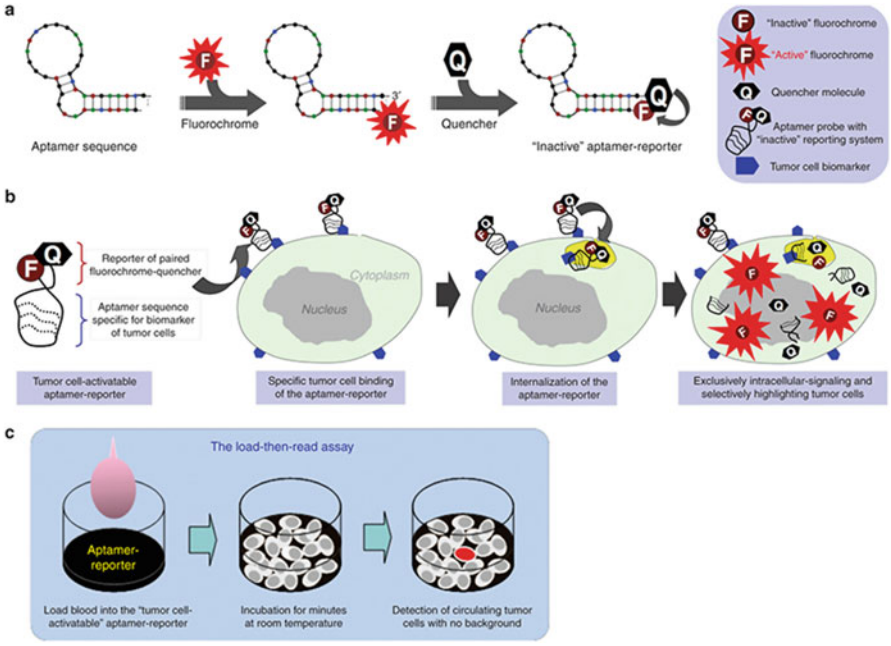

Fig. 3.8 Scheme of the tumor cell-activatable aptamer-reporter for one-step assay of CTCs in a whole blood sample (**a**) A biomarker-specific and tumor cell-activatable aptamer-reporter (**b**) Tumor cell-triggered intracellular activation of the aptamer-reporter. In assays containing tumor cells (**c**) Proposed one-step assay for rapid detection of CTCs (From Zeng et al. 2014, with permission)

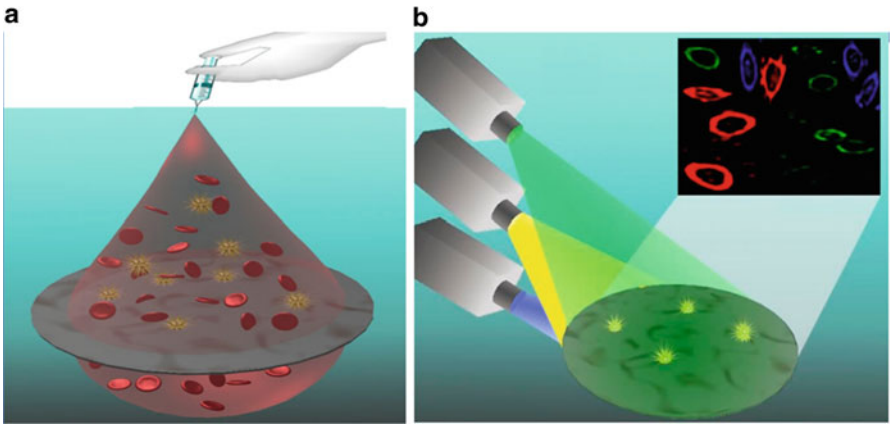

Fig. 3.9 Scheme of aptamer-conjugated porous graphene oxide membrane-based separation and (**a**) Scheme of aptamer-conjugated porous graphene oxide membrane-based capture of multiple CTC types from blood (**b**) fluorescence imaging of multiple types of CTCs captured by graphene oxide membranes using a dye-conjugated aptamer (From Nellore et al. 2015, with permission)

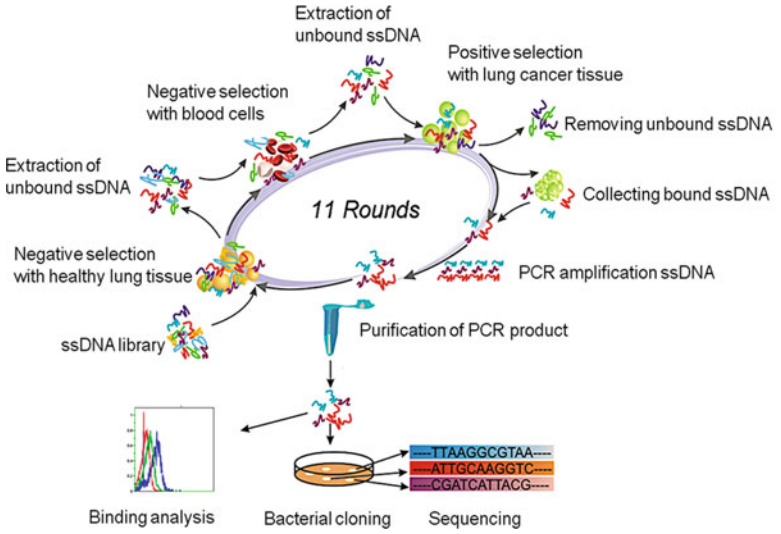

Fig. 3.10 Scheme of aptamer selection to lung adenocarcinoma postoperative tissues for producing of aptamers to intact heterogenic tumor cells (From Zamay et al. 2015, with permission)

ductal carcinomas showed fluorescence staining in the nuclei of the various human cancer cell lines as well as in CTCs isolated from pancreatic cancer patients. This aptamer method was compared with the well-established the anti-cytokeratin method on 15 pancreatic cancer patient blood samples, and enumeration indicated no difference between these two methods.

The unique feature of the work reported by Zamay et al. is the aptamer selection strategy for producing the aptamers for heterogenic lung cancer cell markers in their native state and conformation without previous knowledge of the biomarkers (Zamay et al. 2015). Tissue SELEX was used to select the aptamers with high selectivity to adenocarcinoma derived from postoperative tissues and cells capable to identify various cellular biomarkers (Fig. 3.10).

Interestingly the aptamers had very low affinity to A549 lung adenocarcinoma culture and did not bind to normal lung cells and lymphocytes. Aptamer-associated protein biomarkers for lung cancer for were identified using affinity purification with aptamer-coated magnetic beads followed by the mass-spectrometric identification. Thus anti-vimentin, anti-annexin A2, anti-annexin A5, anti-histone 2B, anti-neutrophil defensin, and anti-clusterin aptamers were used to detect CTCs in blood. These aptamers detected not only CTCs but also apoptotic bodies, and microemboli in clinical samples of peripheral blood of lung cancer and metastatic lung cancer patients. Due to the binding of multiple aptamers to different cell biomarkers, a pool of aptamer clones is more selective and efficient in CTC detection, than a single aptamer or a monoclonal antibody. Application of aptamers in combination with antibodies to tumor-specific antigens provides more reliable detection of rare CTCs. Such tumor-specific aptamers can be produced for individual patients and

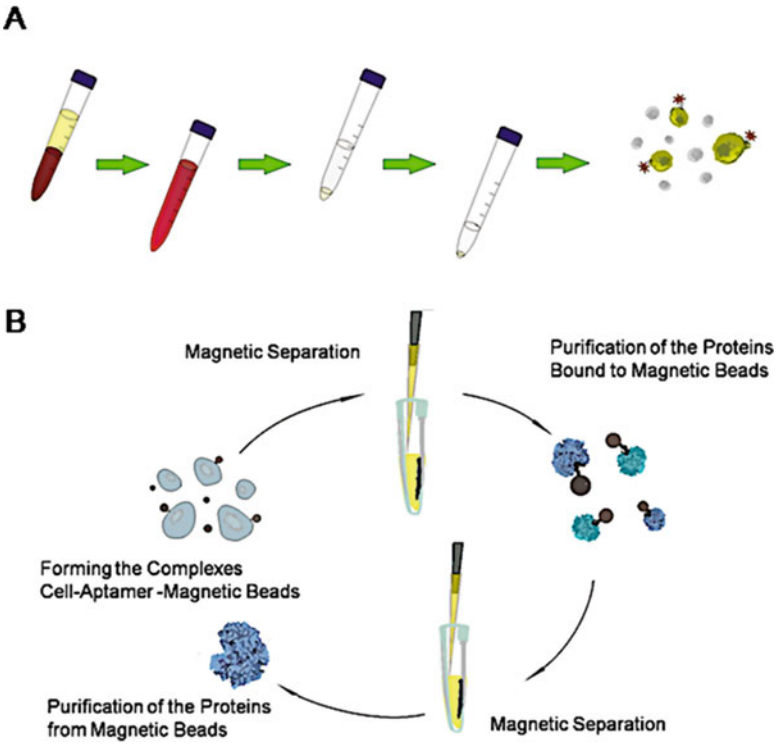

Fig. 3.11 Schemes of blood sample preparation and protein isolation (**a**) Red blood cells were lysed with hypotonic NH_4Cl solution followed by incubation with hypotonic NaCl (**b**) Aptamer-mediated affinity purification of proteins using magnetic separation for further mass spec identification (From Zamay et al. 2015, with permission)

synthesized many times during anticancer therapy, thereby opening up the possibility of personalized diagnostics.

Another valuable finding in this work was an improved blood preparation procedure reducing the time and labour required for the search of rare CTCs (Fig. 3.11). All red blood cells were lysed with NH_4Cl solution and the majority of white blood cells were lysed with hypotonic NaCl solution. Captured CTCs could be used for the following protein, genetic analyses, aptamer associated protein targets identification using aptamer-mediated affinity purification of the target proteins. Interestingly that aptamer-associated proteins identified from adenocarcinoma tissues were similar to the proteins from CTCs.

The captured CTCs pellet was visualized fluorescently labeled aptamers and/or antibodies. Live CTCs were analysed by confocal fluorescent microscopy immediately, or after fixation on glass slide. The additional Romanowsky-Giemsa staining on fixed smears allowed seeing the nuclei and aptamers (Fig. 3.12). Authors analysed blood smears from 105 individuals: 18 healthy people and 87 patients with various diagnosis including different types of primary lung cancer (52),

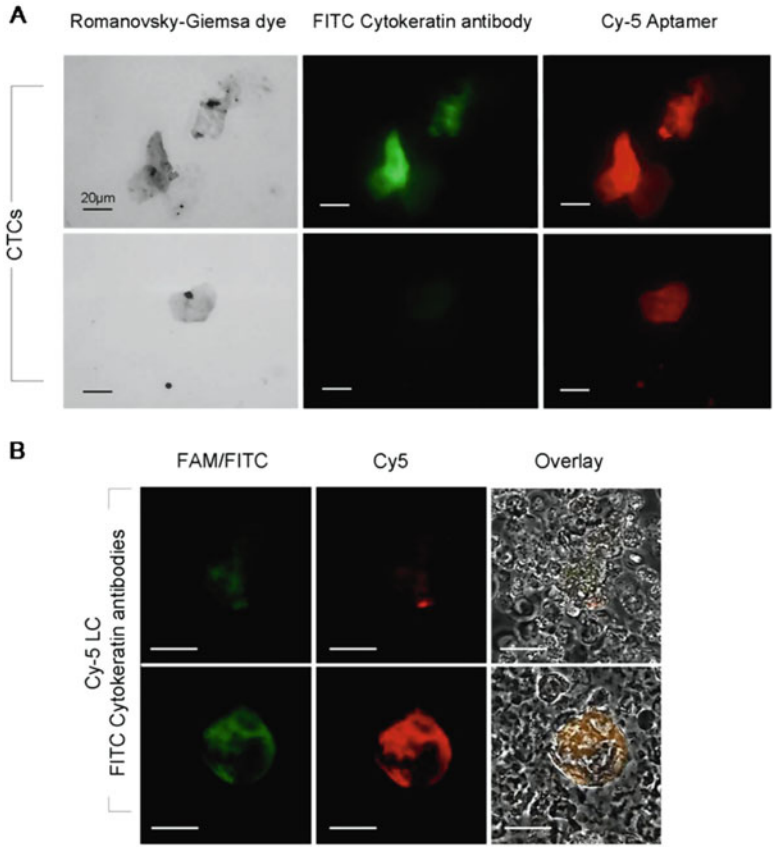

Fig. 3.12 (**a**) Co-staining of live CTCs from blood of a patient with squamous lung cancer by Cy-5 labeled LC-18 aptamer and FITC-labeled anti-pan cytokeratin antibodies (**b**) Fluorescent microscopy of blood smears of lung adenocarcinoma. Samples were pre-incubated with masking DNA, Cy-5 labeled LC-18 and FITC-labeled anti-pan cytokeratin antibodies. The samples were spread evenly on a glass slide. The smears were fixed in methanol for 5 min and then stained with Romanowsky-Giemsa dye. Magnification ×60 (From Zamay et al. 2015, with permission)

secondary lung cancer (1), other lung diseases (9), breast diseases (9), and glioblastoma (16). The sensitivity and specificity of the aptamer – based method was 86% and 76%, respectively (Zamay et al. 2015).

3.6 Conclusion

Tumor cells dissemination through the bloodstream is crucial for the metastasis formation and cancer progression. Therefore, analyses of CTC content in blood can be used after minimally invasive liquid biopsy for cancer diagnosis and prognosis.

Cytokeratins and epithelial cell adhesion molecules (EpCAM) are the most common CTC markers (Joosse and Pantel 2013). However, finding additional CTC markers and corresponding probes are in great demand due to high tumor diversity. Aptamers as synthetic affinity probes could be selected to cancer biomarkers in their native state and conformation without previous knowledge of them. These oncomarkers could be identified after the selection by aptamer-mediated affinity purification with magnetic separation and following mass spectrometry-based analysis. Aptamers that can be used for CTC capture and identification have been recently summarized by Dickey and Giangrande in their review (Dickey and Giangrande 2016).

Different methods and strategies have been developed to isolate and identify CTCs, but their efficacy needs to be validated against existing technologies such as antibody-based strategies (CellSearch) and PCR-based strategies (AdnaTest).

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