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Oligonucleotide Aptamers: a Next-Generation Technology for the Capture and Detection of Circulating Tumor Cells

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Abstract

A critical challenge for treating cancer is the early identification of those patients who are at greatest risk of developing metastatic disease. The number of circulating tumor cells (CTCs) in cancer patients has recently been shown to be a valuable (and non-invasively accessible) diagnostic indicator of the state of metastatic disease. CTCs are rare cancer cells found in the blood circulation of cancer patients believed to provide a means of diagnosing the likelihood for metastatic spread and assessing response to therapy in advanced, as well as early stage disease settings. Numerous technical efforts have been made to reliably detect and quantify CTCs, but the development of a universal assay has proven quite difficult. Notable challenges for developing a broadly useful CTC-based diagnostic assay are the development of easy-to-operate methods that 1) are sufficiently sensitive to reliably detect the small number of CTCs that are present in the circulation and 2) can capture the molecular heterogeneity of tumor cells. In this review, we describe recent progress towards the application of synthetic oligonucleotide aptamers as promising, novel, robust tools for the isolation and detection of CTCs. Advantages and challenges of the aptamer approach are also discussed.

1. Introduction

Cancer is the second leading cause of death worldwide and more than 90% of cancer deaths are due to metastasis [1]. In its early stage cancer is a localized disease. However, in many cases, diagnosis of cancer often comes after it has metastasized throughout the body, thereby hindering the effectiveness of treatment options. Metastasis occurs when cells derived from the primary tumor are shed and transition to a more invasive state prior to gaining access to the circulatory system (Figure 1) [2, 3]. Cancer cells that access the circulation are referred to as circulating tumor cells or CTCs. Recently, counting CTCs has been considered a breakthrough as one way to perform non-invasive monitoring of cancer. Indeed, the number of CTCs present in peripheral blood in multiple types of cancer has been found to strongly

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correlate with cancer patient survival [3, 4]. For example, patients with no CTCs were found to have a better overall prognosis and lower likelihood for metastasis formation compared to CTC-positive patients [5, 6]. Based on these findings, CTCs are thought to provide a means of diagnosing the likelihood for metastatic spread and for assessing response to therapy in advanced, as well as early stage disease settings [3, 7–11].

Numerous technical efforts have thus been made over the past decade to reliably detect and quantify CTCs. However, the development of a universal assay has proven quite difficult as a result of CTCs being very rare events, occurring at rates as low as one cell per 10^6 or 10^7 leukocytes [12]. This is often referred to as *‘the needle in the haystack problem’*. Most methods rely on the combination of two steps, that is, enrichment followed by detection to increase the sensitivity of the assay [13]. Another major technical challenge concerns tumor heterogeneity. Gene-expression profiling has highlighted the remarkable heterogeneity of malignant cells not only within a given histological subtype but also among tumor cells of any given patient. In addition to genetic instability inherent to most neoplastic cell types, emerging data suggest that cell-biological changes during metastatic progression, such as the transition between epithelial-to-mesenchymal states, can also generate multiple distinct cellular sub-populations contributing to intratumoral heterogeneity [14–16]. Owing to this heterogeneity, the assay used for CTC detection could be strongly impacted by the biomarkers present in/on the CTCs, and data regarding CTCs should therefore be interpreted with this in mind.

1.1 CTC capture methods

To enhance sensitivity of CTC detection assays, CTCs are enriched from blood with purification methods prior to detection. Several methods have been developed for capture of CTCs (see Table 1) [17]. These include: **1) *filter-based methods***, which capture cancer cells based on size (e.g. Rarecells and ScreenCell). An example is the ISET (isolation by size of epithelial tumor cells) method from Rarecells [4, 18, 19]. Enriched cells are stained on the filter for cytomorphological examination or further characterized by immunocytochemistry. **2) *microfluidics devices*** (e.g. ApoCell, Creatv Microtech, Fluxion Biosciences, Clearbridge Biomedics), which generally perform enrichment of CTCs based on cellular properties such as size, fluorescent or magnetic labeling of biomarkers on the surface of cells, electrophoretic mobility, and/or cellular adhesion [20]. **3) *fiber-optic array scanning methods*** (e.g. SRI International), which differentiate cancer cells from other blood cells based on their morphology and dielectric properties and **4) *antibody capture methods***, which rely on the expression of cancer cell-specific surface biomarkers (e.g. CellSearch) [21].

1.2 CTC detection methods

(See Table 1 for a comprehensive list of current CTC capture/detection methods). The only FDA approved method for the capture and detection of CTCs is the standardized and semi-automated CellSearch platform (Veridex, Raritan, NJ) [21]. CTC enrichment by CellSearch is based on the expression of the epithelial-lineage marker EpCAM (epithelial cell adhesion molecule). EpCAM-positive cells are enriched by immunomagnetic separation using EpCAM-specific antibodies conjugated to magnetic particles and then stained with fluorescent anti-cytokeratin and 4',6-diamino-2-phenylindole (DAPI), while hematopoietic

cells are stained with anti-CD45 antibodies. Cytokeratin and DAPI-positive, and CD45-negative CTCs are finally counted by using a semi-automated fluorescent microscope [5, 22–24]. Although effective, the paucity of molecular markers for screening CTCs is a true drawback of this technology, limiting its application to just a few tumor types with high EpCAM expression [25–28]. While carcinomas are epithelial in origin, the expression of this surface marker on CTCs can vary greatly. Indeed, it has been reported that less than 30% of breast CTCs express EpCAM due to the epithelial-to-mesenchymal transition [27–29].

Other capture methods (e.g. ApoCell) suffer from the same limitations due to their reliance on antibody-based or other conventional assays for CTC detection/analysis. ApoCell's technology, called ApoStream, relies on dielectrophoretic field flow fractionation to separate CTCs from the blood in a microfluidic flow chamber [30]. This method relies on the unique density and dielectric properties of CTCs compared to normal blood cells. However, after isolation of the CTCs ApoStream is still reliant on current CTC detection methods in order to enumerate and characterize the CTCs in the specimen [30].

Additional CTC detection methods include PCR of CTC-specific DNA or RNA sequences (Biocept, Cynvenio, Fluxion, Panomics). These approaches rely on microfluidic or filter capture technologies followed by an RNA or DNA purification step prior to performing downstream analysis (based on next-generation sequencing (NGS) and Affymetrix technologies). The advantages of these methods include high sensitivity and broad applicability. The obvious drawbacks include long detection/processing times, difficulty of implementation in most clinical diagnostic labs (requires microfluidic and NGS expertise and infrastructure), and high cost per sample (kits to perform isolation and RNA hybridization/amplification steps). Other fee-for-service technologies like those offered by Epic Sciences, SRI International and CellSearch have similar advantages and drawbacks. Given the limitations of existing detection methods/services, novel technologies are being evaluated for the development of the next generation of non-invasive cancer diagnostics.

2. Nucleic Acid Aptamers

Nucleic acid aptamers have been proposed as a new potential solution to overcoming many of the drawbacks of current CTC detection assays. Some of the anticipated advantages of aptamer-based assays include ease of implementation, robustness (sensitivity and specificity) and speed. In addition, because aptamers can be rapidly developed against most targets, their implementation is likely to overcome the challenges of tumor heterogeneity.

Aptamers are synthetic oligonucleotide ligands with affinities and specificities for their target comparable to those of antibody/antigen interactions [31]. Aptamers are derived from combinatorial sequence libraries (with complexities of up to $\sim 10^{14}$) by a process termed SELEX (Systematic Evolution of Ligands by EXponential Enrichment) (Figure 2) [32, 33]. The library of aptamers is screened against proteins or cells of interest in order to select aptamers with desired properties of protein binding and/or internalization in cells. Following each round of selection, the remaining aptamers are cloned and can be screened repeatedly over multiple rounds to select the best aptamers for the given selective pressures.

Additionally, negative selection pressures can be applied to remove aptamers that bind to proteins or cells to which binding is not wanted. Aptamers can be made from RNA or DNA, and are oftentimes modified to make them resistant to nuclease-mediated degradation by using nucleosides such as 2'-fluoro-pyrimidines and 2'-O-methyl-pyrimidines during the selection process. These modified RNA or DNA aptamers can then be used in cell culture as well as *in vivo* in animals [34].

Over the past decade, this technology has been used to generate high-affinity RNAs to a large number of proteins. Aptamers and their targets have been extensively reviewed over the last several years [35–43]. Given their properties, aptamers are quickly emerging as powerful new therapeutic and diagnostic tools [36, 39–50]. Of relevance to CTC capture and detection are aptamers to cell surface biomarkers. Aptamers have been developed to a multitude of cancer cell surface biomarkers over the years, including PSMA, HER2, CEA, and EpCAM, among others, and have been used for several purposes, such as chemotherapeutic drug delivery and siRNA delivery, which has been extensively reviewed [37–43, 51–55]. Aptamers have also been developed that bind to cancer cells using cell-SELEX, however many of their receptors are not yet known.

The use of aptamers for the isolation and/or detection of CTCs has recently been investigated by several groups. Aptamers have been used to develop more efficient methods of CTC isolation/detection, and to identify new biomarkers for cancer. Below we summarize current progress in the aptamer field towards developing robust reagents for the capture and detection of CTCs (Table 2).

3. Aptamer-based technologies for CTC enrichment/capture

Because of the difficulty of isolating and enriching CTCs from the blood with existing technologies, several groups have sought to develop strategies based on aptamer technology to improve capture efficiency and biomarker recognition. These strategies involve immobilizing high affinity aptamers onto various material surfaces and using them as capture agents for CTCs. A comprehensive summary of the various aptamers and aptamer technologies used for CTC capture is provided below.

3.1 Aptamer-coated microfluidic technology

Phillips et al. was the first group to report using an aptamer-coated microfluidic device for the enrichment of cancer cells in 2009 (Figure 3A) [56]. This work used the Sgc8 aptamer, which was previously selected using cell-SELEX to bind to CCRF-CEM cells [57]. The aptamer was immobilized upon a polydimethylsiloxane (PDMS) channel and a mixture of CCRF-CEM cells (which bind to Sgc8), and a negative control cell line, NB-4 (which does not bind to Sgc8), were pumped through the channel, and the target cells were captured with high efficiency and purity. The group expanded upon this preliminary work in a study by Xu et al. later that same year [58]. They showed that the device was capable of enriching multiple different target cancer cells (suspended in buffer) into independent fractions simultaneously, by immobilizing three different aptamers onto different sections of the microfluidic device. The aptamers that were evaluated included TD05 (anti-B-Cell Receptor), Sgc8 (anti-CCRF-CEM cells), and Sgd5 (anti-Toledo cells), previously

developed using cell-based SELEX protocols [57, 59]. Importantly, the authors observed a > 130 fold enrichment of the target cells using this approach. While promising, these studies await validation of enrichment of cancer cells from blood and patient samples.

3.2 Aptamer micropillar-base microfluidic technology

A variation on the aptamer microfluidics capture approach by Xu et al. is an aptamer micropillar-based microfluidic device pioneered by Sheng et al. [60]. The aptamer micropillar device contains over 59,000 elliptical micropillars with dimensions of $30\mu\text{m}$ (major axis) $\times$ $15\mu\text{m}$ (minor axis) $\times$ $32\mu\text{m}$ (height), and an interpillar distance of $80\mu\text{m}$. The micropillars and flow channels are coated with DNA aptamers previously selected against cancer cell lines: CCRF-CEM cells, Ramos cells, HCT 116 cells, and DLD-1 cells [57, 61–63]. This micropillar design favored high affinity interactions between the aptamers and the cancer cells, and yielded a capture efficiency of approximately 95% and purity of approximately 81%. The device is approximately the size of a microscope slide, and it is able to isolate as few as 10 colorectal tumor cells from 1 milliliter of unprocessed whole blood in less than 30 minutes. Another advantage of this approach is that approximately 93% of the captured cancer cells remain viable and can subsequently undergo further cellular and molecular characterization. This device has advantages such as rapid analysis, no need to pre-treat blood samples, and a low limit of detection, and has the potential to be useful in clinical applications for patients with cancer, such as diagnosis, prognosis, and monitoring the response to treatments.

3.3 Hele-Shaw microfluidic technology

To efficiently capture rare cancer cells in solution, Wan et al. developed a Hele-Shaw microfluidic device containing glass beads functionalized with an RNA aptamer which binds to Epidermal Growth Factor Receptor (EGFR) [64–66]. The beads were arranged in an ordered array of pits within a PDMS channel. When solutions containing cancer cells were flowed through the channel, the beads captured cancer cells with high specificity. After capture of the cancer cells, a solution containing RNA complementary to the aptamer sequence was used to release the cells from the beads, and by using this approach, the cells remained viable for further downstream analysis. Although the authors used cells isolated from patient tumor samples, future work will need to focus on applying this approach to capture CTCs from the blood of patients.

3.4 Aptamer-functionalized biochips

Anti-EGFR aptamers have also been used in a unique way by Wang et al. This group generated a biochip with RNA aptamers coating a microelectrode on a silicon dioxide layer [67]. The biochip contains a PDMS cover with a channel for introducing cells. The anti-EGFR aptamers specifically bind the tumor cells, allowing the measurement of the resistance and detection of a single cell between the electrodes, which had never been done with aptamers to detect cancer cells before. In future work, this technique will need to be used to isolate CTCs from blood, however, if successful, it could eventually be incorporated with other lab-on-a-chip components for further downstream analysis of CTCs.

3.5 Aptamer-functionalized gold nanoparticle technology

Liu et al. were the first to use aptamer-functionalized gold nanoparticle to enrich and detect cancer cells (Figure 3B) [68]. The authors used a pair of aptamers - a thiolated aptamer (TD05) and a biotinylated aptamer (TE02) - which were both selected by cell-SELEX to bind to Ramos cells in previous work [59]. The thiolated TD05 was immobilized on gold nanoparticles, and the biotinylated TE02 was immobilized in a region, or “test zone,” upon a nitrocellulose membrane, termed an “aptamer-nanoparticle strip biosensor” (ANSB) within a lateral flow device. After the Ramos cells interacted with the TD05 conjugated to the gold nanoparticles, cell-TD05-gold nanoparticle complexes flowed down the ANSB and accumulated on the test zone by binding to the TE02 aptamer. The gold nanoparticles could then be visualized and quantified, as they produced a visible red band. One drawback of this approach is that larger volumes of blood (> 5 μ L) mask the signal from the cancer cells. This was due to the non-specific adsorption of erythrocytes on the membrane, potentially restricting its usefulness for clinical applications. Optimizations of this technology are justified for future clinical implementation.

Aptamer-functionalized gold nanoparticle technology has also been combined with microfluidics to further enhance capture of rare cancer cells from blood [69]. In a recent study by Sheng et al., the authors conjugated up to 95 Sgc8 aptamers to gold nanoparticles to promote highly efficient binding of CCRF-CEM cells. A laminar flow flat channel microfluidic device was then used to capture the bound CCRF-CEM cells from blood. The authors were the first to demonstrate that combining aptamer-functionalized nanoparticle technology with microfluidics can greatly enhance the robustness of aptamer-based capture CTC methods enabling the future implementation of aptamers as cancer diagnostics.

3.6 Aptamer-coated silicon nanowire technology

Shen et al. developed aptamer-coated silicon nanowire substrates to immobilize cancer cells within a stationary device, termed a “NanoVelcro” cell affinity substrate [70]. DNA aptamers were screened against A549 non-small cell lung cancer cells using cell-based SELEX, and two resulting selected aptamers were used to coat the NanoVelcro chip. After enriching the A549 cells from blood, the NanoVelcro chip was treated with nuclease to digest the aptamers and release the captured cells, resulting in the recovery of approximately 75% of the 200 A549 cells which had been spiked into 1 milliliter of blood, as well as 300 to 1,500 white blood cells. The NanoVelcro chip enabled efficient capture of A549 cells from whole blood and subsequent release following nuclease treatment. An advantage of this technique is the high viability of the recovered cancer cells, which allows for further downstream characterization of the cells. Another advantage of this approach is that removing the bound aptamers with the nuclease avoids altering downstream signaling pathways, resulting in the ability to further characterize captured cancer cells in a more native state. Future work will need to focus on enriching CTCs from cancer patient blood samples, and, if the device is able to achieve sufficient CTC enrichment, it could potentially be utilized in conjunction with established CTC detection techniques for use in the clinic.

3.7 Aptamer-functionalized graphene oxide membrane capture technology

Viraka Nellore et al. used an RNA aptamer-modified graphene oxide membrane that has pores with a diameter of 20 to 40 μm to capture and identify multiple types of cancer cell lines (including breast, prostate, and colon cancer lines) from blood (Figure 3C) [71]. The efficiency of capture was approximately 95%, and this technique showed effectiveness with as few as 10 cancer cells per milliliter of blood. In this study, 3 different RNA aptamers were used that bind various targets including the S6 aptamer which binds human epidermal growth factor receptor 2 (HER2), the A9 aptamer which binds prostate-specific membrane antigen (PSMA), and the YJ-1 aptamer which binds carcinoembryonic antigen (CEA) [51–53]. In addition to binding their targets, the A9 and YJ-1 aptamers have functional effects of inhibition of the migration of tumor cells expressing PSMA and CEA respectively [51, 53]. The aptamers each had a different fluorescent dye conjugated to the 5' end, allowing the different fluorescent labels to be utilized to identify different cell types using multicolor fluorescence imaging. In the future, this technique could be utilized for capturing CTCs in patient blood samples, as well as a platform for the simultaneous analysis of different CTC types based on the fluorescence signature of the aptamers.

3.8 Aptamer-functionalized hydrogel technology

In a recent study by Li et al., the Sgc8 aptamer described above was conjugated to hydrogels (aptamer-functionalized hydrogels) to specifically capture CCRF-CEM cancer cells (Figure 3D) [72]. The group coated small squares of silanized glass surfaces in polyacrylamide hydrogels containing an acrydite-conjugated DNA oligonucleotide linker attached to the hydrogel. The hydrogel-coated glass was then incubated in an aptamer solution to immobilize the DNA aptamer to the complementary linker DNA sequence embedded within the hydrogel. Subsequently, the coated glass squares were incubated in a cell suspension to bind the target cells, followed by removal of the unbound cells by a 1 minute shaking step. Of note, following efficient capture of the cancer cells, the authors introduce a wash step with endonucleases to degrade the aptamers. This step enabled the facile release of cancer cells for further processing/characterization, without compromising their viability. These aptamer-functionalized hydrogels still need to be validated in blood. However, these hydrogels have the potential to improve medical devices (such as microfluidic devices) through their use as a coating which could allow for the specific capture and release of CTCs without harming the cells.

3.9 Aptamer-coated dish capture technology

EpCAM antibodies are used in the only FDA-approved CTC detection assay by CellSearch [21]. Like antibodies, anti-EpCAM aptamers have also shown promise for use in the capture of CTCs. Song et al. used an *in vitro* SELEX protocol to select a DNA aptamer which binds to EpCAM [54]. The anti-EpCAM aptamer (SYL3C) was able to selectively bind EpCAM-positive cancer cells and not bind EpCAM-negative cells. Biotin-labelled SYL3C was attached and immobilized on a streptavidin coated dish, followed by incubation with a mixture of EpCAM-positive and EpCAM-negative cells, allowing specific capture of the EpCAM-positive cells. Future work will need to focus on capturing EpCAM-positive CTCs from patient blood samples, and comparing the efficacy of this method to the established

CellSearch method. This technique could offer improvements over CellSearch due to several advantages of aptamers over antibodies, such as their smaller size, easier synthesis, and increased stability while still maintaining high affinity and specificity of binding to EpCAM.

3.10 Aptamer-gold nanofilm capture technology

A unique strategy was used by Chiu et al., who described the enrichment of MCF-7 breast cancer cells from blood using Mucin1-binding aptamer-modified gold nanofilms (Figure 3E) [73, 74]. Following cancer cell enrichment, pulsed laser desorption/ionization mass spectrometry (LDI-MS) was used to detect gold cluster ions from the nanofilms. When MCF-7 cells were spiked into blood from a healthy donor, as few as 500 cancer cells per milliliter blood could be detected. This is the first study which used LDI-MS for the detection of CTCs, but because the limit of detection was 500 cancer cells per milliliter of blood, there needs to be improvement in the sensitivity for this to be used in the clinic. However, this technique employs a high throughput LDI-MS analysis and could be useful for the diagnosis of cancer and in monitoring the effectiveness of treatments in cancer patients.

4. Aptamer-based technologies for CTC detection

4.1 Aptamer fluorochrome-quencher detection technology

Aptamers have been used to detect cancer cell lines spiked into blood and/or CTCs in several different ways. One interesting strategy, described by Zeng et al., used an anti-CD30 RNA aptamer conjugated to paired fluorochrome-quencher molecules, which do not emit fluorescence in the absence of tumor cells (Figure 4a) [75, 76]. This aptamer-based CTC probe specifically interacts with cell surface biomarker proteins on cancer cells and is internalized and trafficked to the lysosome where it is degraded. Degradation of the aptamer results in separation of the fluorochrome and the quencher, thereby allowing the fluorochrome to emit fluorescence. This was successfully used to identify CTCs in the whole blood of lymphoma patients, with little-to-no off-target signal from the blood cells. A strong advantage of this strategy is that it allows for the processing of whole blood from patients with just a single step in order to detect CTCs. This approach has a high potential to be used as a simple test for the early detection of CTCs in the blood of patients for diagnosing cancer and monitoring the progression of treatment.

4.2 Aptamer-functionalized spherical colloidal crystal cluster technology

Zheng et al. recently described a novel strategy whereby cancer cells spiked into blood can be captured and detected with DNA aptamer-functionalized spherical colloidal crystal clusters (Figure 4B) [77]. The reflective properties of the colloidal crystal structures, which can be controlled during their fabrication, allows for their use as “barcode particles.” These specifically varied reflective properties are utilized as a way of detecting and evaluating the identity of the particular particles (e.g. reading the barcodes). The authors used the TD05 (anti-B-Cell Receptor), Sgc8 (anti-CCRF-CEM cells), and Sgd5 (anti-Toledo cells) aptamers, which had previously been developed using cell-based SELEX protocols [57, 59]. The three different aptamers were linked to three different barcode particles, allowing for the capture, detection, and subsequent release of multiple different types of cancer cells from

the particles. Although the technique has shown promise with blood samples containing spiked cancer cells, the effectiveness of this technology still needs to be proven in cancer patient blood samples. However, this novel platform holds great promise for simultaneous, high throughput enrichment and detection of multiple different kinds of CTCs expressing different biomarkers in patients.

5. Aptamer-based technologies for novel CTC biomarker identification

5.1 Cell-SELEX

In a recent study published in 2015, Zmay et al. validated the use of cell-SELEX for developing aptamers against cancer cells for which there are no known biomarkers. The authors selected DNA aptamers to lung adenocarcinoma cells derived from postoperative patient tissues (Figure 2A) [78]. Negative selections were performed to remove aptamers that bind to healthy lung tissues and healthy blood in order to increase the specificity of the aptamers for lung cancer cells. With this strategy, the aptamers were selected specifically for lung cancer cell biomarkers in their native state and conformation without prior knowledge of the biomarkers. Zmay et al. used the selected aptamers to detect CTCs in the peripheral blood of lung cancer and metastatic lung cancer patients. Importantly, this strategy resulted in the identification of novel protein biomarkers for lung cancer that were targeted by the aptamers, including annexin A2, annexin A5, cathepsin D, clusterin, histone 2B, neutrophil defensin, and vimentin. This approach could allow for the selection of tumor-specific aptamers for individual patients, and the selected aptamers could be utilized as a tool to monitor the progress of treatments, thereby offering personalized diagnostics.

5.2 In situ tissue slide-based SELEX

Zhang et al. developed a novel *in situ* tissue slide-based SELEX strategy, which used a positive selection to identify DNA aptamers that bind to formalin-fixed, paraffin-embedded breast infiltrating ductal carcinomas (Figure 2B) [79]. Additionally, the authors performed a negative selection against adjacent normal breast tissue to remove aptamers that bind to targets on normal breast tissue. Using this strategy, the authors selected an aptamer (BC-15) which they found was able to bind cells lines derived from several different kinds of cancer (pancreatic adenocarcinoma, breast cancer, lung carcinoma, and colon adenocarcinoma). They showed fluorescence staining with BC-15 in the nuclei of the various human cancer cell lines as well as in CTCs isolated from pancreatic cancer patients, and saw that binding of the aptamer was much lower in normal breast epithelial cells and leukocytes. The authors demonstrated that the BC-15 aptamer was equally effective at CTC enumeration as compared to an established anti-cytokeratin staining method using antibodies in 15 pancreatic cancer patient blood samples. Finally, they utilized mass spectrometry to identify the binding target of BC-15 as heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), which had previously been identified as a good potential biomarker for early detection of multiple types of cancer [80]. Similar to the study by Zmay et al., this work shows that aptamers can be selected to an individual's tumor sample, opening up the possibility of personalized diagnostics. These strategies offer advantages over the use of antibodies, by allowing the detection of heterogeneous and/or non-immunogenic biomarkers of the individual patient's tumor cells.

6. Discussion

The interest in CTCs from a research and clinical perspective has been growing rapidly due to studies implicating CTCs in metastatic development and clinical diagnostics. However, the paucity of robust tools for capture and analysis of these rare cells have significantly hindered the study and broad application of CTCs for cancer diagnostics. The only FDA-approved method and much of the focus of current research strategies for isolating and enumerating CTCs relies on antibody-based technologies which, have several drawbacks for many diagnostic and clinical applications. In 2008 it was reported that half of all commercially available antibodies are nonspecific and do not selectively bind to their intended targets [81]. Even more concerning is the idea that the results of 47 out of 53 high-impact preclinical studies were unable to be replicated largely due to inadequately characterized antibodies [82, 83]. Here we summarize recent advances in aptamer technology towards the capture and detection of CTCs in blood.

Because aptamers bind their targets with affinities similar to antibodies (low pM to nM range) they are logical substitutes for any application that uses antibodies. However, aptamers offer several advantages over antibodies. For instance, aptamers can easily be forced to release the bound target without harsh treatments such as high heat, salt concentrations, or pH, thus preserving the integrity of the target [84]. For example, the introduction of a sequence complementary to the aptamer sequence can be enough to compete off the binding of the target [85]. Aptamers can also be removed with wash steps that include chelators (e.g. EDTA) or endonucleases (e.g. RNaseA) [70, 72, 86]. In order for an antibody to release its target protein, oftentimes harsh chemical or enzymatic treatments are used, which can be harmful to cells [87].

Another important property of aptamers is that they can be selected/designed to be inert capture agents that will not interfere with cell-surface receptor signaling [39, 88–90]. This is important for downstream applications where further characterization of CTCs, such as determining gene expression profiles of the cells, is desired. Indeed, bivalent antibodies are known to disrupt downstream pathways often resulting in altered cell growth or viability of the cells [91, 92]. As antibodies cannot be easily removed from cells, they interfere with downstream genotypic and phenotypic analyses.

As discussed above, a major technical challenge to the development of a universal assay to reliably capture/detect rare cancer cells in blood concerns tumor heterogeneity. Towards this end, the SELEX methodology has been adapted to isolate aptamers against most targets even without prior knowledge of a given target (e.g. cell-SELEX has been used to identify novel biomarkers expressed on cancer cells and CTCs) [39, 41, 43, 78, 79]. Complex aptamer libraries have been screened against various cancer cell lines to enable the identification of aptamers that bind novel biomarkers in the context of the cell membrane. Importantly, features of these selections include stringent preclear steps (usually performed against other non-cancer cells or blood cells) to enhance the specificity of the selected aptamers. Using similar selection approaches, aptamers have also been selected against non-immunogenic targets, thereby increasing the repertoire of target cancer biomarkers that are often missed using antibody technology [38].

While aptamer-based technologies hold great potential for the capture and detection of CTCs, they have yet to be evaluated against existing technologies such as antibody-based strategies (CellSearch) and PCR-based strategies (AdnaTest), thus warranting more thorough experimentation [93–96]. Future efforts focused on thoroughly evaluating the benefits of combining different technologies (e.g. aptamer capture technology and microfluidics or filter capture methods with aptamer detection technology) will also facilitate the implementation of this technology for cancer diagnostics. Additionally, aptamers against several different cancer cell biomarkers (PSMA, Her2, etc.) could easily be used in combination with the current technology developed by CellSearch to capture and enumerate a wider range of cancer types simultaneously.

In conclusion, aptamers have demonstrated great flexibility by being integrated within and improving upon numerous previously-established CTC isolation and detection strategies. Aptamers offer enormous potential for targeting known cancer biomarkers as well as aiding in the discovery of novel biomarkers. Moreover, the prospect of personalized diagnostics using aptamer technology is very exciting. Accessing the information contained within CTCs will be extremely helpful for diagnosing and treating cancer in patients. For instance, cancer could be diagnosed at an early stage if a routine blood test could be performed during regular doctor visits to screen for the presence of CTCs. Furthermore, characterizing the mutations and the gene expression profiles of the CTCs could provide insights into what the best treatment strategies are for a patient, and continually monitoring CTC levels over time could glean valuable information about the effectiveness of the treatments. With further research and optimization, the aptamer-based technologies discussed here may provide the key to unlocking that invaluable information.

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Abbreviations

CTC	Circulating tumor cell
FDA	Federal Drug Administration
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
DAPI	4',6-diamino-2 phenylindole
NGS	Next-generation sequencing
SELEX	Systematic evolution of ligands by exponential enrichment
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid

PSMA	Prostate specific membrane antigen
HER2	Human epidermal growth factor receptor 2
CEA	Carcinoembryonic antigen
hnRNP A1	Heterogeneous nuclear ribonucleoprotein A1
PDMS	Polydimethylsiloxane
ANSB	Aptamer-nanoparticle strip biosensor;

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Highlights

- Circulating tumor cells (CTCs) are important for the metastatic spread of cancer.
- Isolating and characterizing CTCs could provide valuable clinical information.
- Technologies to isolate and study CTCs have been extensively investigated.
- Aptamers have recently emerged as new and powerful tools to study CTCs.
- Here we provide a review on the use of aptamers in CTC research.

Figure 1. Model of circulating tumor cells and cancer metastasis. Cells derived from a primary, localized tumor are shed, transition to a more invasive state, and enter the circulatory system. Once inside the circulatory system, these circulating tumor cells can extravasate and form metastatic tumors at distant sites in the body.

A. Cell-SELEX

B. In situ tissue slide-SELEX

Figure 2.

Cell-SELEX and *in situ* tissue slide-SELEX protocols. (A) In traditional cell-SELEX, an RNA aptamer library is transcribed from a DNA library. The aptamers are subjected to a negative selection on non-target cells to remove aptamers with undesirable qualities such as binding and internalization in the non-target cells. The aptamers that do not bind/internalize in the non-target cells are then subjected to a positive selection on target cells to find aptamers with desirable qualities (such as binding/internalization in the target cells). The aptamers are then purified from the cells and screened over multiple rounds until the best aptamers are selected. (B) Zhang et al. describe an *in situ* tissue slide-based SELEX strategy where a DNA aptamer library is initially negatively selected using slides containing normal breast tissue to remove aptamers that bind to targets on normal breast tissue. The aptamers that do not bind the normal tissue are then positively selected against breast infiltrating ductal carcinomas.

Figure 3.

Aptamer-based technologies for CTC enrichment/capture. (A) General overview of microfluidic devices containing aptamers which are used for enriching CTCs from the blood. (B) TD05 aptamers are immobilized on gold nanoparticles and biotinylated TE02 are immobilized in a “test zone” upon a nitrocellulose membrane. CTCs are coated by the TD05-gold nanoparticle conjugate and are captured by binding to the TE02 aptamer within the test zone. Visualization of the TD05-gold nanoparticle conjugate allows for the detection of the captured CTCs. (C) An RNA aptamer-modified three dimensional graphene oxide membrane containing pores with a diameter of 20 to 40 μm captures and identifies breast, prostate, and colon cancer cell lines from blood. Three different aptamers are labeled with three different fluorophores, allowing the identification of what type of cancer cells was captured. (D) Silanized glass surfaces are coated with polyacrylamide hydrogels attached to an acrydite-conjugated DNA oligonucleotide linker within the hydrogel. The hydrogel-coated glass is then incubated in an aptamer solution to immobilize the DNA aptamer to the complementary linker DNA embedded within the hydrogel. The coated glass squares are then incubated in a cell suspension to bind the target cells. Washing with restriction endonucleases degrades the aptamers and releases the CTCs. (E) Gold nanofilms coated with a DNA aptamer that binds Mucin1 are used to enrich CTCs from blood. Pulsed laser desorption/ionization mass spectrometry (LDI-MS) is then used to detect gold cluster ions from the nanofilms. When CTCs are bound by the aptamers the number of gold cluster ions detected is decreased compared to when no CTCs are bound.

A. Aptamer fluorochrome-quencher detection

B. Aptamer-functionalized spherical colloidal crystals

Figure 4.

Aptamer-based technologies for CTC detection. (A) An aptamer labeled with a quencher and a fluorophore is used as a probe to specifically interact with cell surface biomarker proteins on CTCs. The aptamer is internalized, trafficked to the lysosome, and degraded. The degradation of the aptamer results in separation of the fluorochrome and the quencher, thereby allowing the unquenched fluorochrome to emit fluorescence, which can be used to detect the CTCs using fluorescence microscopy. (B) Cancer cells are captured from blood and detected with DNA aptamer-functionalized spherical colloidal crystal clusters. The

reflective properties of the colloidal crystal structures allows for their use as “barcode particles,” to detect and evaluate the identity of the particles (e.g. reading the barcodes).

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Table 1

Commercially available CTC capture and detection technologies

Company Name	Capture Technology	Detection/Analysis Technology
Biocept http://biocept.com/	Purification of DNA from blood	High throughput sequencing platform
Cynvenio – ClearID platform http://www.cynvenio.com/	Microfluidic platform (based on CK+/CD45– selection)	High throughput sequencing platform
Fluxion Biosciences http://fluxionbio.com/	IsoFlux microfluidic platform	High throughput sequencing platform
Apocell http://www.apocell.com/	Microfluidic platform	Standard assays
Clearbridge Biomedics http://www.clearbridgebiomedics.com/	Microfluidic platform	Standard assays
Biocept http://biocept.com/	Antibody platform	Standard assays
CellSearch https://www.cellsearchctc.com/	Antibody platform (based on EpCAM+, CK+, CD45–)	Standard assays
Creatv Microtech http://www.creatvmicrotech.com/	Filter technology	
Rarecells http://www.rarecells.com/	ISET filter technology	
ScreenCell http://www.screencell.com/	Filter technology	
Panomics/Affymetrix http://www.panomics.com/products/rna-in-situ-analysis/ctc-platform/overview	ScreenCell filter technology	Affy microarray technology
Epic Sciences http://www.epicsciences.com/	Multiple platforms (proprietary)	Multiple platforms (proprietary)
SRI International http://www.sri.com/research-development/rare-cell-technology		Fiber-optic scanning technology

Table 2

Aptamers used for CTC capture and/or detection

Biomarker/ Cell Line	Cancer Types	Aptamer Name(s)	SELEX Strategy	RNA or DNA	Aptamer Modifications	Capture or Detection	Ref.
EGFR	Colorectal, Glioblastoma, Lung	Anti-EGFR Aptamer	Recombinant protein	RNA	2' Fluoro- Pyrimidine	Capture	[64–67]
EpCAM	Adenocarcinoma	SYL3C	Recombinant protein	DNA	Truncated	Capture	[54]
Her2	Breast	S6	Cell-based (SK-BR-3 Cells)	RNA	-	Capture and Detection	[71]
PSMA	Prostate	A9	Recombinant protein	RNA	2' Fluoro- Pyrimidine	Capture and Detection	[71]
CEA	Breast, Colorectal, Gastric, Lung, Medullary Thyroid, Pancreatic	YJ-1	Recombinant protein	RNA	-	Capture and Detection	[71]
Mucin1	Breast, Gastric, Ovarian	APT _{MUC}	Recombinant protein	DNA	-	Capture	[73]
CD30	Lymphoma	Apt1	Recombinant protein	RNA	-	Detection	[75]
B-Cell Receptor	Lymphoma	TD05	Cell-based (Ramos Cells)	DNA	-	Capture and Detection	[58, 60, 68, 77]
Ramos Cells	Lymphoma	TE02	Cell-based (Ramos Cells)	DNA	-	Capture and Detection	[68]
CCRF-CEM Cells	Leukemia	Sgc8	Cell-based (CCRF-CEM Cells)	DNA	-	Capture and Detection	[56, 58, 60, 69, 72, 77]
Toledo Cells	Lymphoma	Sgd5	Cell-based (Toledo Cells)	DNA	-	Capture and Detection	[58, 77]
A549 Cells	Non-Small Cell Lung Carcinoma	Ap-1 and Ap-2	Cell-based (A549 Cells)	DNA	-	Capture	[70]
hnRNP A1	Breast, Colon, Lung, Pancreatic	BC-15	Cell-based (<i>in situ</i> Breast Cancer Tissue Slides)	DNA	-	Detection	[79]
HCT 116 Cells	Colorectal	KDED2a-3	Cell-based (HCT 116 Cells)	DNA	Truncated	Capture	[60]

Biomarker/ Cell Line	Cancer Types	Aptamer Name(s)	SELEX Strategy	RNA or DNA	Aptamer Modifications	Capture or Detection	Ref.
DLD-1 Cells	Colorectal	KCHA10	Cell-based (DLD-1 Cells)	DNA	-	Capture	[60]
Annexin A2	Breast, Laryngeal Carcinoma, Lung, Pancreatic	LC-17	Cell-based (Lung Cancer Tissue)	DNA	-	Detection	[78]
Annexin A5	Breast, Laryngeal Carcinoma, Lung, Pancreatic	LC-17	Cell-based (Lung Cancer Tissue)	DNA	-	Detection	[78]
Vimentin	Breast, Colorectal, CNS, Endometrial, Hepatic, Lung, Pancreatic, Prostate	LC-17	Cell-based (Lung Cancer Tissue)	DNA	-	Detection	[78]
Neutrophil Defensin 1 and 3	Lung	LC-18	Cell-based (Lung Cancer Tissue)	DNA	-	Detection	[78]
Histone H2B	Lung	LC-18 and LC-110	Cell-based (Lung Cancer Tissue)	DNA	-	Detection	[78]
Clusterin	Lung	LC-110	Cell-based (Lung Cancer Tissue)	DNA	-	Detection	[78]
Cathepsin D	Breast, Colorectal, Lung	LC-183	Cell-based (Lung Cancer Tissue)	DNA	-	Detection	[78]