Advanced Biosystems

Microfluidic Technology for Nucleic Acid Aptamer Evolution and Application

--Manuscript Draft--

Manuscript Number:	adbi.201900012R1
Full Title:	Microfluidic Technology for Nucleic Acid Aptamer Evolution and Application
Article Type:	Invited Review
Section/Category:	
Keywords:	
Corresponding Author:	Julian Alexander Tanner, Ph.D. University of Hong Kong Pokfulam, HONG KONG
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Hong Kong
Corresponding Author's Secondary Institution:	
First Author:	Lewis A. Fraser
First Author Secondary Information:	
Order of Authors:	Lewis A. Fraser
	Yee-Wai Cheung
	Andrew Kinghorn
	Wei Guo
	Simon C.C. Shiu
	Chandra Jinata
	Mengping Liu
	Soubhagya Bhuyan
	Lang Nan
	Ho Cheung Shum
	Julian Alexander Tanner, Ph.D.
Order of Authors Secondary Information:	
Abstract:	The intersection of microfluidics and aptamer technologies holds particular promise for rapid progress in a plethora of applications across biomedical science and other areas. Here, we review the influence of microfluidics on the aptamer field, from traditional capillary electrophoresis approaches through to innovative modern-day approaches using micromagnetic beads and emulsion droplets. Miniaturising aptamer-based bioassays through microfluidics has the potential to transform diagnostics and embedded biosensing in coming years.
Additional Information:	
Question	**Response**
Please submit a plain text version of your cover letter here.	We submit the minor revision of the invited review article entitled “Microfluidic technology for nucleic acid aptamer evolution and application” for consideration by Advanced Biosystems. We have addressed all the reviewer comments. We look forward to hearing from you,

	Julian Tanner (on behalf of all authors)
Do you or any of your co-authors have a conflict of interest to declare?	No. The authors declare no conflict of interest.

DOI: 10.1002/ ((please add manuscript number))
Article type: Review

Microfluidic Technology for Nucleic Acid Aptamer Evolution and Application

Lewis A. Fraser, Yee-Wai Cheung, Andrew B. Kinghorn, Wei Guo, Simon Chi-Chin Shiu, Chandra Jinata, Mengping Liu, Soubhagya Bhuyan, Lang Nan, Ho Cheung Shum , Julian A. Tanner**

Dr. L. A. Fraser, Dr. Y.W. Cheung, Dr. A.B. Kinghorn, S.C.C.Shiu,, C. Jinata, M. Liu, Dr. J.A.Tanner
School of Biomedical Sciences
LKS Faculty of Medicine
The University of Hong Kong
Hong Kong (SAR), China
Email: jatanner@hku.hk

Dr. W. Guo , L. Nan, Dr. H.C. Shum
Department of Mechanical Engineering
Faculty of Engineering
The University of Hong Kong
Hong Kong (SAR), China
Email: ashum@hku.hk

Keywords: Microfluidics, Aptamers, Selection, SELEX, Biosensors.

Abstract

The intersection of microfluidics and aptamer technologies holds particular promise for rapid progress in a plethora of applications across biomedical science and other areas. Here, we review the influence of microfluidics on the aptamer field, from traditional capillary electrophoresis approaches through to innovative modern-day approaches using micromagnetic beads and emulsion droplets. Miniaturising aptamer-based bioassays through microfluidics has the potential to transform diagnostics and embedded biosensing in coming years.

1. Introduction

This review will focus on the cross-section of two rapidly developing areas: nucleic acid aptamers, and microfluidics. The microfluidic selection of aptamers will encompass solution-phase, bead-based and droplet-based microfluidic aptamer selections. Additionally, aptamer-based microfluidic sensors will be discussed including biosensors, whole cell biosensing, protein sensing, small molecule detection and nanostructure-mediated sensing.

1.1 Aptamers

Nucleic acids are generally regarded as the polymeric molecules that store and pass genetic information across generations, and enable protein production. Across biology, it is now clear that nucleic acids play a plethora of roles in a number of processes. Across biotechnology and nanotechnology, there has been major progress in use of nucleic acids for a wide range of applications. One of these technologies has been in approaches for the *in vitro* evolution and application of nucleic acid aptamers. Aptamers are short, single-stranded nucleic acids which are capable of specific high affinity binding. Binding is facilitated by various noncovalent interactions including hydrogen bonding, van der Waals interactions, electrostatic interactions, and broadly by shape complementarity. Aptamers can bind a wide range of targets including various biological macromolecules, whole cells, surfaces or small molecules. Systematic evolution of ligands by exponential enrichment (SELEX) is the method by which aptamers are isolated, originally developed by the laboratories of Ellington, Szostak and Tuerk, Gold.[1] SELEX is an iterative process that involves selection of nucleic acids from a massive library of random sequence oligonucleotides for a particular characteristic, typically binding. In each SELEX round the oligonucleotide library is incubated with that target molecule followed by wash steps to remove non-binding aptamers, leaving just the tightly bound aptamers that are subsequently amplified by PCR. The amplified strands from the previous round form a new

enriched library used in subsequent rounds of SELEX. Several rounds of SELEX enrichment result in an aptamer library that tightly binds to the target of interest. These aptamers are sequenced and characterised for affinity and specificity. Aptamers can be selected with high-affinity K_d values in the pM to nM range.[2] Relative to their often contrasted counterpart protein antibodies, nucleic aptamers can be simply synthesised through solid phase synthesis with little batch-to-batch variation, and typically have higher thermal stability.[3]

The aptamer field has had its share of successes and challenges. One notable success was the development of the Food and Drug Administration (FDA) approved aptameric drug known as "Macugen" which targets the angiogenic cytokine vascular endothelial growth factor (VEGF) associated with macular degeneration.[4] Aptamers have had some impact on diagnostics,[5] such as the DNA aptamer developed against botulinum toxin.[6] Aptamer potential has been demonstrated in drug detection, such as the 'split' aptamer sensitive for cocaine in human blood serum.[7] The Jaffrey lab has developed fluorescent RNA for live-cell imaging and aptamer-targeted antibody delivery.[8] Additionally, aptamers have been integrated as functional modular controllers of DNA nanostructures.[9] Other emerging approaches include the use of an extended genetic code, xeno-nucleic acids (XNAs) which has resulted in the first synthetic genetic catalysts (XNAzymes) first described by the Holliger group.[10]

Technologies for the directed evolution of nucleic acids aptamers by SELEX have shown remarkable diversity, and microfluidics has had a significant impact in recent years. Capillary electrophoresis was the first microfluidic technique to influence aptamer selection.[11] Subsequently, high throughput techniques using magnetic beads and emulsions in microfluidics were developed.[12] Emulsion-based techniques encapsulate the individual oligonucleotide sequences within single droplets, known as clonal droplets.[13] After clonal droplet generation, emulsion PCR can be used such that droplets containing single oligonucleotides undergo thermocycling to result in populations with droplets each containing

a clonal expansion of the single parent oligonucleotide .[13] Droplets have substantially lower rates of cross-reactivity and amplicon generation, which is ideal for the generation of aptamers.[13-14] Furthermore, new droplet based microfluidic techniques allow the selection of nucleic acids based on their intrinsic catalytic activity.[15] Aptamers generated by SELEX have been functionalised in a range of microfluidic biosensors.[16] These approaches have increased the portability, automation, throughput and sensitivity of various aptamer-based assays, thus enhancing aptamer-based biosensing.

1.2 Microfluidics

As a technology to manipulate and control liquid and particles in micro channels, microfluidics has had impact across a range of fields including biology, chemistry and materials science.[17] Since the development of microfluidics in the early 1990s, numerous techniques have been developed to realise multiple functions, including rapid reactions, precise liquid manipulation, and inter-chip analysis of target samples.[18]

The most basic function of microfluidics is to speed up reactions within small volumes. At the micron and submicron scales of microfluidic channels, the surface-to-volume ratio is several orders higher than in bulk systems, resulting in more atomic exposure during reactions.[19] A reduction in the length scale will dramatically accelerate the diffusion process with a time scale of $\tau_d \sim l^2/D$, where D is the diffusion coefficient of the samples. Thus, more efficient chemical and biological reaction times are achieved.[20] Active mechanical forces, such as acoustic actuation and electrokinetic actuation, are often implemented into the channel to accelerate mixing, further decreasing reaction times.[21] Moreover, droplet microfluidics segments the continuous flow of a bulk solution into discrete volumes in the form of droplets. Droplets can be sorted with a frequency of up to several kilohertz, thus allowing high-throughput operations and preventing cross-contamination.[22]

In addition to utilizing the natural advantages of microfluidics, passive structures and active interventions are increasingly imposed in microfluidic channels to allow manipulation of liquid samples. Passive techniques mainly comprise of trapping structures for immobilisation,[23] and can be coupled to specific channel geometries for sample processing, such as cell separation and cell lysis.[24] These methods are considered simple and convenient to implement, but there can be issues with robustness and efficiency due to the lack of control.[25] In comparison, active methods, whereby external forces are applied such as dielectrophoresis (DEP), magnetic force, optical tweezers and surface acoustic waves (SAW) are more controllable, precise, and enable programmable manipulation at high temporal and spatial resolutions.[26] Furthermore, with the advent of droplet technologies, different microfluidic modules can be combined to realise a series of droplet-based sample manipulations, from target encapsulation, polymerase chain reaction (PCR) to droplet sorting.[27]

Advanced methods for the detection and analysis of desired targets can be performed in microfluidic channels. A series of detection techniques can be implemented into microfluidics to examine both morphologies and contents of droplets. Optical imaging is a direct observation method, aiming at monitoring morphological change of droplets under physiological conditions.[28] Similarly, fluorescence detection can be performed to quantify the intensity of a reaction and to determine the concentration of specific molecules.[29] More sophisticated detection methods are based on additional measurement elements. For example, the utilisation of deposited electrodes and adherent biological probes to detect current variation and concentration of specific molecules.[30] Additionally, force sensor and on-chip probes are frequently used to measure the stiffness, deformability, and mechanical properties of target containing samples.[31]

2 Microfluidic selection of aptamers

The *in vitro* selection of aptamers is achieved using SELEX, which consists of iterative rounds of target incubation, partitioning and amplification.[14] Although conventional SELEX protocols are straightforward and well-established, alternative selection approaches are continuously being developed. Alternative methods are required as conventional SELEX has relatively poor partitioning, can be time and labour consuming, and is typically only capable of selection for binding and simple single-turnover reactions. The partitioning stage is critical for aptamer selection, therefore techniques with high separation efficiency which allow for selection of exotic properties are of great utility.

2.1 Solution phase microfluidic aptamer selection

Capillary electrophoresis (CE) is a microfluidic approach developed in the early 1980's as an alternative analytical technique to gel electrophoresis and liquid chromatography.[11, 32] The separation of molecules by using CE mainly relies on two mechanisms, electrophoretic mobility and electroosmotic flow, within a capillary with a charged inner wall. Electrophoretic mobility fractionates molecules based on their electrophoretic force, whereas electroosmotic flow is driven by the difference in potential across the capillary wall and the molecules. Usually electroosmotic flow is a stronger force than electrophoretic mobility, therefore net migration is typically towards the cathode. CE was used for the partitioning step in SELEX, a method accordingly termed CE-SELEX.[33] Aptamers against immunoglobulin E (IgE) were identified in just four rounds and showed nanomolar affinity to their selection target. As ten or more rounds of conventional SELEX are required to isolate aptamers, this significant reduction in selection rounds demonstrated the efficiency of applying CE to aptamer selection. An alternative application of CE, so-called non-equilibrium capillary electrophoresis of equilibrium mixture (NECEEM) was also demonstrated (Figure 1a).[34] NECEEM differs from CE-SELEX in that no amplification steps are used between CE partitioning rounds. NECEEM is a one-round selection approach that involves several rounds of partitioning by CE and only

a single round of PCR amplification at the end of the selection process.[35] Based on the success of NECEEM-based aptamer selection, many more aptamers have been identified, including aptamers against thermostable DNA mismatch binding protein (MutS),[36] signal transduction proteins,[37] bovine catalase and tau protein.[38]

Although NECEEM has allowed for aptamer selection using only a single PCR amplification step after the partitioning stage, one drawback is the very low amount of template collected from CE. This makes it difficult to obtain a high PCR yield without contamination by non-specific amplicons. Emulsion PCR (ePCR) is a technique in which a PCR reaction mixture is compartmentalized into a water in oil emulsion. The compartmentalization of reagent droplets in ePCR means that deleterious non-specific amplicons are contained and cannot spread and consume PCR products in other droplets. The addition of ePCR to NECEEM further improved this aptamer selection technique. Krylov and colleagues have demonstrated the power of ePCR incorporated NECEEM by selecting aptamers against an unstable protein, AlkB homologue 2, which is a very challenging target for conventional SELEX.[39] The significant reduction in selection rounds demonstrated the utility of applying CE to aptamer selection.

2.2 Bead-based microfluidic aptamer selection

Magnetic beads have been widely implemented for the solid support immobilization of molecular targets in SELEX since their first use in 1997.[40] The principle behind magnetic bead-based SELEX partitioning is very simple. In brief, targets covalently immobilized to micro-beads are used to separate target binding DNA of interest. However, in terms of the separation efficiency and practicality, magnetic bead based separation is inferior to other partitioning approaches such as capillary electrophoresis.[41] To address this issue, novel magnetic SELEX techniques were developed that make use of microfluidic systems.[12]

The Soh group pioneered the integration of magnetic bead-based SELEX with microfluidic systems. They developed a continuous-flow magnetic activated chip-based separation

(CMACS) device (Figure 1b).[12] In this chip, the aptamer library is bound to the target immobilized beads which are guided by a magnetic field to travel along a nickel surface to a product outlet. The unbound aptamers were collected through waste outlets. In just a single round of selection using this device, an aptamer pool with a K_d value of 33 nM against the light chain of recombinant *Botulinum* neurotoxin type A was isolated. Though the results were promising, the CMACS device was limited in some respects by delicate manual performance and flow stream disruptions caused by microbubbles or blockages. [12]

To address these issues, Soh *et al.*, 2009 developed another microfluidic device called a Micro-Magnetic Separation (MMS) chip.[42] Compared to the CMACS device, this MMP chip was fabricated with grids of ferromagnetic materials (titanium and nickel) producing magnetic field gradients that can be magnetized externally. With this device, aptamers that targeted the streptavidin-conjugated magnetic beads were first captured in the channel by application of an external magnetic field, whilst unbound aptamers were removed by a continuous washing buffer stream. Afterwards, the adhered nucleic acids could be released and collected through the outlet by removing the magnet field. Aptamers against streptavidin were obtained within three positive rounds of selection, and their specificities were further enhanced through another round of negative selection. So far, this MMS chip had exhibited comparable or better recovery (~99.5 %) and partition efficiency (10^6) when compared to those achieved in the CMACS chip.[12] Additionally, this method was faster as it allowed higher flow rates (>10 mL/h). Furthermore, it was the first study that integrated both positive and negative selections in one microfluidic device. Based on this chip, Soh *et al.*, 2010 selected aptamers against PDGF-BB and streptavidin through introducing some optimizations such as highly stringent selection conditions,[43] which resulted in aptamers with higher affinity and specificity. Similarly, Wang *et al.*, 2014 developed optimized magnetic beads-assisted microfluidic systems for screening aptamers against myoglobin, which integrated both positive and negative selections.[44] While

the Soh group performed the two types of selections individually,[12] Wang *et al.*'s chip was able to perform a positive selection directly after negative selection. After seven rounds of selection, aptamers against myoglobin with bulk K_d value ranging from 4.93 to 6.38 nM were successfully screened.

The above-mentioned bead-based microfluidic selection methods have focused on the partitioning step. SELEX consists of many other steps including target-aptamer library incubation, PCR amplification, and affinity/specificity evaluations. Microfluidic systems have potential for highly integrated SELEX in a single chip so the next stage was to develop novel microbead-based microfluidic SELEX platforms integrated with multiple functional units.

The first fully SELEX integrated microfluidic system was developed by the Gwo-Bin Lee group. Inspired by the automatic microfluidic SELEX system proposed by Hybarger *et al.* and the bead-based microfluidic system developed by Soh *et al.*[12, 45], They fabricated a microfluidic system comprising of three functional units; a microfluidic control unit, a bead-based aptamer extraction unit and a PCR unit.[46] This platform performed target-aptamer incubation, aptamer separation and on-chip PCR amplification in parallel. An aptamer was successfully screened against C-reactive protein (CRP) with K_d value as low as 3.51 nM in a rapid manner (60 min/round). Lee *et al.* further optimized their integrated magnetic chip by coupling it with a competitive assay chip, which improved the selection as it enabled immediate affinity and specificity measurements of the aptamer pool directly after extraction.[47] The entire SELEX process took only 20 min which represents a dramatic reduction to time and cost of an aptamer selection. Using a similar technique, Lin *et al.* screened two specific aptamers in just 70 min against hemoglobin A1c and hemoglobin with K_d values at 7.6 nM and 7.3 nM respectively.[48] Subsequently, an integrated microfluidic system was developed by Kim *et al.* that coupled the affinity selection and amplification step by an electrophoretic oligonucleotide manipulation scheme. This allowed for the rapid selection of aptamers against either surface-

immobilized protein (immunoglobulin E) or solution-phase small molecule (bisboronic acid-glucose mixture) with high affinity.[49]

Bead-based microfluidic aptamer selection makes SELEX more rapid, efficient and ultimately lowers the costs. One drawback of the previously mentioned bead-based microfluidic aptamer selection systems is that the aptamer extraction step cannot be monitored on chip, therefore selection round failure would not be detected until all SELEX rounds are complete. To overcome this problem, Hong *et al.*, 2017 combined the selection chip with a fluorescent signal evaluation system, which monitors target-coated magnetic nanospheres.[50] The ssDNA aptamer pools were FAM-labelled such that when they were incubated with targets in the evaluation chip for 2 hours at a low flow rate, the fluorescent intensity of bound DNA could be measured to monitor bulk binding affinity *in situ.* , Hong *et al.,* 2019 improved upon this microfluidic selection setup by efficiency via trapping magnetic nanospheres on microscale.[51] It took just three selection rounds to select nanomolar dissociation constant aptamers against Ebola antigens.[51]

Another potential drawback with magnetic bead immobilization is the nonspecific interactions of oligonucleotides to the bead surface. Carboxylic acid-coated beads that are negatively charged, and hence electrostatically repel DNA, have been useful in addressing the nonspecific binding problem.[12] Despite these drawbacks, bead-based microfluidic aptamer selection greatly enhances partitioning and streamlines the selection process when compared to conventional SELEX.

2.3 Droplet based microfluidic aptamer selection

In vitro compartmentalization (IVC) is a powerful tool, creating clonal droplets and facilitating high throughput single molecule species analysis. This technology is especially useful in directed molecular evolution. IVC has applications in the directed molecular evolution of enzymes and functional nucleic acids.[15, 52] In this section we will focus on IVC impact on the

directed evolution of functional nucleic acids in the form of aptamers and ribozymes. Generally nucleic acid aptamers have been selected for binding. However, more complex characteristics have been selected such as fluorogenicity and catalysis including nuclease and Diels-Alder cycloaddition activity.[15, 52c, 53] IVC directed evolution is particularly suited to selection for these activities as the compartmentalization links a genotype with a phenotype, consisting of sequence information and activity respectively.

RNA aptamers against fluorogenic compounds are useful for RNA imaging in cells as well as sensing and diagnostics. Paige *et al.*, 2011 used binding capture selection to isolate RNA aptamers against the fluorogenic compound DFHBI.[53] When DFHBI is in its native state no fluorescence is observed, however when its aptamer is bound the complex exhibits a strong fluorescent signal. The brightest aptamer isolated against DFHBI was termed "Spinach" due to the green fluorescent signal produced. The spinach aptamer was used for RNA tracking in cells,[53] detection of metabolites and as a split aptamer.[54]

Using the Spinach aptamer sequence as a starting point, Autour *et al.*, 2016 performed microfluidic directed evolution to improve the spinach aptamer's performance.[13] A total of five rounds of microfluidic selection were performed: two enrichment rounds followed by one mutation round and then two enrichment rounds. A selection round consisted of emulsification of PCR reaction mix containing the aptamer library in a Poisson distribution, thermal cycling of PCR droplets, fusion of PCR droplets with *in vitro* transcription droplets containing DFHBI fluorogen, incubation and FADS (Figure 1c). Autour *et al.* selected aptamers that displayed fluorescent activity in a variety of salt conditions including K^+, Na^+, Li^+, Cs^+ and no salt. [13] The relative molar fluorescence of the DFHBI/iSpinach complex was greater than that of DFHBI/Spinach2 by 1.4 times in K^+ buffer and by 2.1 times in Na^+ buffer. [13]

Dolgosheina *et al.*, 2014 used binding capture selection to isolate RNA aptamers against the fluorogenic compound Thiazole Orange.[55] This fluorescence upon binding aptamer was

termed “Mango”. As for the Spinach aptamer fluorophore pair, when Thiazole Orange is in its native state no fluorescence is observed, however when the Mango aptamer is bound the complex exhibits a strong fluorescent signal. To isolate aptamers against Thiazole Orange, twelve rounds of classical SELEX were performed. Interestingly the resulting aptamers bound to biotinylated Thiazole Orange but not native Thiazole Orange. [55] The Mango aptamer/fluorogen pair was used for single molecule imaging, was expressed in bacteria, and injected into *Caenorhabditis elegans* for imaging. [55]

Autour *et al.*, 2018 used the 12th round library from the work of Dolgosheina *et al.*, 2014 and performed 10 rounds of FADS selection.[56] One limitation of FADS is that 100 nM of TO1-B was required for effective fluorescent detection of the droplets. The dissociation constant of the original aptamer was 3.2 ± 0.7 nM, so improving upon aptamer dissociation constant by decreasing TO1-B concentration was not feasible. Instead, a competition assay was used to challenge the aptamer/TO1-B interaction with N-methyl mesoporphyrin IX (NMM) and TO3-B. For the 1st to 4th selection rounds, NMM from 3 μM to 8 μM was used and for the 2nd to 9th selection rounds TO3-Biotin was used from 3.5 nM to 230 nM. [56] In addition to molecular competition, the stability of the RNA/TO1-B complex was challenged by sorting the droplets at 45 °C. The resulting aptamers exhibited fluorescence signal eight times greater than that of the original Mango aptamer when measured at 37 °C. [56]

An important reaction in synthetic chemistry is the Diels-Alder [4 + 2] cycloaddition reaction,[15] which occurs between a 1,3 diene and an alkene dienophile. IVC was used to select for ribozymes that catalyse multiple-turnover Diels–Alder cycloadditions. [15] The selection strategy was to covalently label an entire ribozyme gene library with anthracene, a Diels–Alder cycloaddition substrate. After IVC of single DNA templates and transcription, any active ribozyme would react the supplemented biotin-maleimide to the library linked anthracene, thereby labelling the ribozyme encoding DNA with biotin. Since the IVC isolates each library

member and its respective ribozymes, there is no cross reactivity between different library members and ribozymes and the connection between genotype (DNA) and phenotype (ribozyme activity) is covalently linked. The emulsion is then broken and the biotinylated DNA is isolated using streptavidin coated para-magnetic beads, before PCR amplification. To achieve multiple-turnover selection conditions, free anthracene is emulsified with the library. After eleven rounds of selection nearly all ribozymes contain a common catalytic motif with similar activity. The best ribozyme had a turnover rate (k_{cat}) of 0.33 ± 0.06 s^{-1}. [15] Although the actual partitioning step was not performed in a microfluidic device, the association of biotin with DNA led to selection of higher performance ribozymes such that the selection step was performed within droplets.

Using a self-cleaving RNA SELEX selection method, Tang and Breaker 2000 selected an RNA cleaving ribozyme.[52c] The dominant species isolated from the selection was the X-motif. Although a relatively fast ribozyme, the X-motif's steady state turnover (k_{cat}^{ss}) was ~20-fold lower than the apparent turnover during the pre-steady state burst phase (k_{burst}). This indicates that the rate-limiting step of the reaction is product release. To address the rate-limiting product release of X-motif, Ryckelynck *et al.*, 2015 performed a droplet-based microfluidic selection to select for nuclease activity under multiple turnover conditions. [52c] Cycles of random mutagenesis and selection were performed to evolve the X-motif. The microfluidic selection consisted of PCR mixture emulsification, thermocycling, fusion of *in vitro* transcription mixture with PCR droplets, incubation to allow for transcription, pico-injection of activity assay mixture, incubation to allow assay development and fluorescence activated sorting. After nine rounds of microfluidic selection they obtained X-motif variants with a steady state turnover (k_{cat}^{ss}) ~28-fold higher than the original X-motif. [52c] This increase was primarily due to an increase in the rate of product release, which was the original X-motif's rate-limiting step in a multiple-turnover reaction setting.

IVC is a powerful tool which has been used in many research areas. Utilizing IVC for the selection of nucleic acid aptamers has expanded the potential selection repertoire from simple binding and single turnover catalysis to more exotic properties such as turn on fluorescence upon binding and multiple turnover catalysis. A great potential exists for selection for other properties, just so long as the assay can be monitored fluorometrically. IVC technologies such as droplet generation, droplet merger, pico-injection and droplet sorting are under continuous advancement, [13] and will lead to faster, higher throughput selections which will allow for greater sequence space coverage and greater selection success. IVC is ideally suited to nucleic acid aptamer selection.

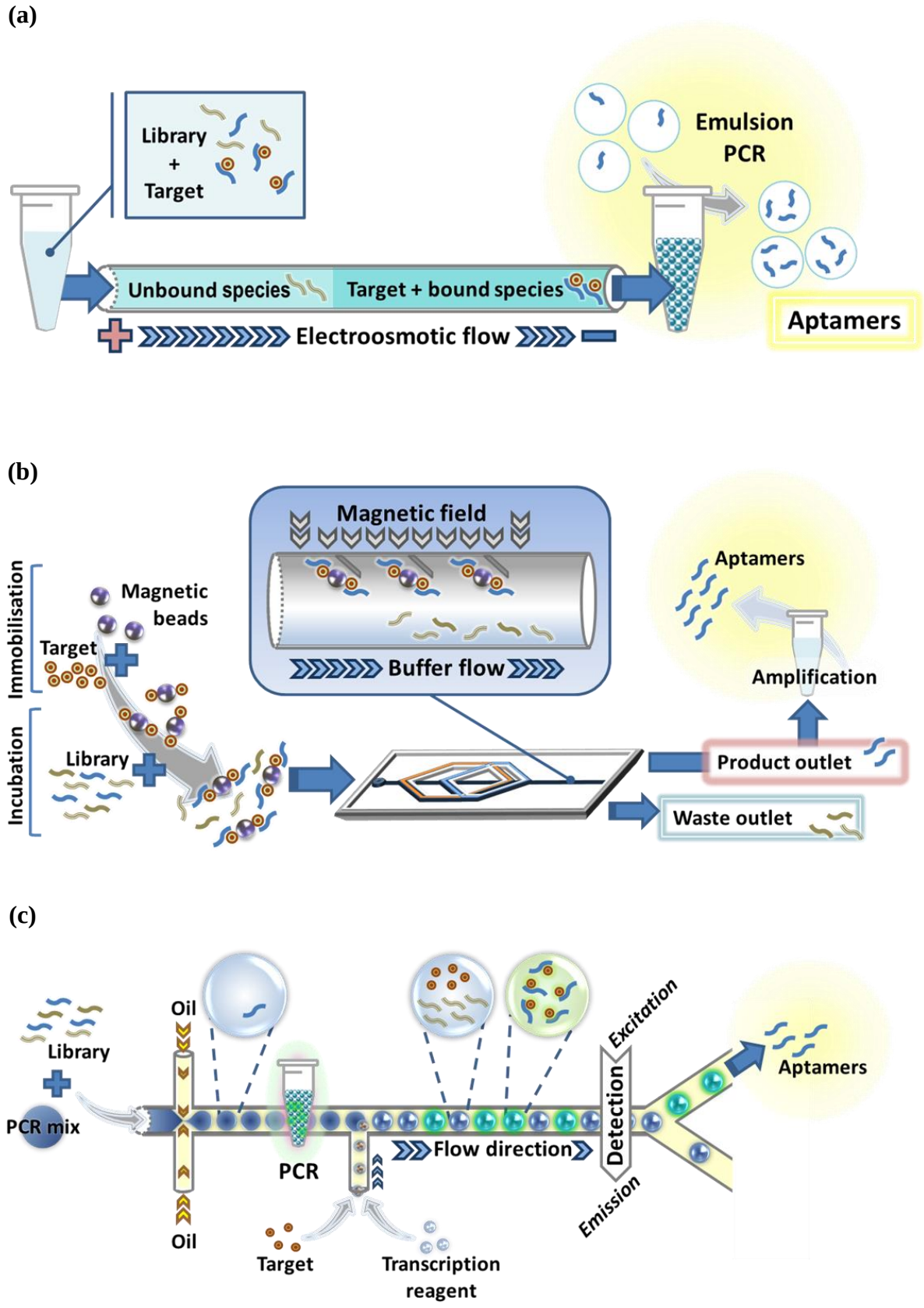
(a)
Library
+
Target
Emulsion
PCR
Unbound species
Target + bound species
Aptamers
Electroosmotic flow
(b)
Magnetic field
Aptamers
Immobilisation
Magnetic
beads
Target
Buffer flow
Amplification
Incubation
Library
Product outlet
Waste outlet
(c)
Oil
Library
Excitation
Aptamers
PCR mix
Detection
Flow direction
PCR
Oil
Emission
Target
Transcription
reagent

Figure 1. Schematic of different microfluidic aptamer selection approaches. a) Solution phase microfluidic aptamer selection. Aptamer library and target are incubated together before being injected into a capillary to undergo non-equilibrium capillary electrophoresis of equilibrium mixture (NECEEM). Target bound species are separated before emulsion PCR (ePCR) for reliable amplification of low abundance template. b) Bead-based microfluidic aptamer selection. Target immobilised to magnetic beads is incubated with the aptamer library. The mixture is then injected into the microfluidic device and the magnetic beads are held in place using an external magnetic field. Aptamers bound to the target-coated beads are guided by Ni strips and eluted through the product outlet, while unbound aptamers are diverted to the waste outlet. The binding aptamers are further transported for PCR amplification. c) Droplet based microfluidic aptamer selection. An aqueous phase made up of DNA library and PCR mix is emulsified within a microfluidic system, before PCR amplification to create monoclonal droplets. These droplets are then merged with droplets containing transcription reagent and fluorogenic target molecule before incubation at 37°C to allow transcription. Droplets then undergo FADS to select for fluorescence-activating RNA aptamers.

3.1 Aptamer based microfluidic biosensors

Aptamer-based microfluidics enable biosensors to sensitively detect a range of biomarkers. Microfluidics are ideal for biosensing as channels use lower sample volumes, which allows for more efficient use of reagents and biological samples. Microfluidic chips can be combined for multiplexed detection of multiple analytes[16a] and flow rates, velocity, and the internal architecture of microfluidic channels can be optimised to improve binding capacity.[16b, 16c] Furthermore, microfluidic channels can be designed to manipulate, process and separate components of complex biological samples,[16d] for example the separation of blood components in live animal blood for detection of small molecule biomarkers.[16e] Additionally, aptamers are perfect candidates for integration into microfluidic biosensors as they are durable

across a range of conditions[3] and are smaller than antibodies, so therefore can be densely packed into microfluidic channels.[57] Here we discuss how aptamer integration with microfluidics has led to highly effective biosensors for the detection of whole cells, proteins and small molecules.

3.2 Whole cell biosensing

A range of aptamer-based microfluidics have been generated for the detection of whole cells. Aptamers can be selected to bind specific proteins on the surface of cell[58] and to proteins in their native state on live cells without previous knowledge of the exact protein biomarker.[59] Furthermore, aptamers can be combined in cell specific 'cocktails' to increase specificity and overcome cellular heterogeneity.[60] The majority of microfluidic cell-based detection reports have focused on the detection of circulating tumour cells (CTCs). However, aptamer-based microfluidic biosensors have also been successfully developed for other cell detection applications, including for the detection of bacterial cells. **Figure 2** presents a generalised scheme which describes the key steps in aptamer-based microfluidic CTC capture biosensors.

3.2.1 Detection of circulating tumour cells

CTCs are cells released into the blood stream by metastatic cancers which are capable of forming secondary metastatic tumours.[61] Measuring CTCs can aid physicians in monitoring progression and recurrence of cancer.[62] The first publication outlining the concept of aptamer-based CTC in a microfluidic was described by the Weihong Tan research group, in which DNA aptamers which bind to T-cell acute lymphocytic leukaemia cells with a K_d of 0.8 nM were conjugated to an avidin-coated PDMS microchannel. [63] Solutions of dyed cells were passed through the microfluidic channel and captured cancer cells were imaged by confocal microscopy. The cancer cells were captured with a >97 % purity and a >80 % efficiency.[63] This initial principle of aptamer capture of CTCs in microfluidic channels has been developed

over the last decade and aptamer based microfluidic CTC capture devices can now selectively capture and release CTCs,[64] and capture and isolate single CTC cells.[65] A range of different CTCs can now be captured in aptamer-based microfluidics (Table 1).

An advantage of using microfluidic devices is the capacity to modify internal architectures for increased binding capacity and processing complex biological solutions. For example, a microfluidic was designed which contained >59,000 aptamer coated micropillars.[65] As a cell containing biological samples passes through this microfluidic the micropillars increase the probability of aptamer cell binding events by a principle known as deterministic-lateral-displacement.[66] This novel internal architecture allows more sample to be rapidly processed without sample pre-treatment. As a result of the micropillars, the device captured 10 tumour cells from 1 mL of whole blood, a >95% capture efficiency.[65]

Many advances in aptamer-based microfluidics for CTC capture have focused on the analysis of cancer cells for research purposes, such as selective capture and release of CTCs, [67] capture and isolation of single CTC cells,[65] and gDNA isolation to examine genetic mutations.[68] However, advances have also complemented the development of biosensors for diagnostic purposes. Fluorescent detection is typically used in CTC biosensing. CTCs are stained by membrane staining dyes or DNA staining dyes and subsequently imaged by confocal microscopy,[69] a method which is less amenable to biosensor portability. However, electrochemical detection systems are portable instrument-free detection systems. For example, a PDMS microfluidic device was reported which uses an electrochemical method containing EGFR binding aptamers and two integrated electrodes. Aptamer-captured CTCs are positioned between the electrodes and specifically detected by impedance measurements.[70] Song *et al.*, 2019 developed a lateral displacement-patterned microfluidic chip modified with multivalent aptamer-functionalized nanospheres to improve CTC capture efficiency.[71] They were able to improve binding efficiency 100-fold, and multivalent capture improved capture efficiency 3-

fold relative to monovalent capture. The captured CTC were released using a thiol exchange reaction with 80% efficiency and 96% cell viability, thus enabling compatibility with downstream cellular analysis.[71]

3.2.2. Detection of non-CTCs

Aptamers can be selected against a broad range of cellular motifs and applied in biosensors. Consequently, they can be used to detect non-CTCs. Biosensor detection of bacterial cells is of particular interest when detecting water and food-based pathogens.[72] A biosensor was developed to detect water-based *Escherichia coli,* in which a solution containing *E.coli* cells was incubated with aptamer coated fluorescent nanoparticles was passed through a PDMS microfluidic.[73] The design used a sheath flow junction to focus the sample into the centre of a microfluidic channel where a laser based optofluidic particle sensor counted bacterial cells coated in the fluorescent nanoparticles. This method was shown to be specific against alternative bacterial strains, and capable of detecting ~100 particles per second with an ~85% accuracy.[73] To our knowledge there is not currently an electrochemical aptamer based microfluidic biosensor for the detection of bacterial cells, though antibody alternatives exist which could benefit from innovations in aptamer research.[74]

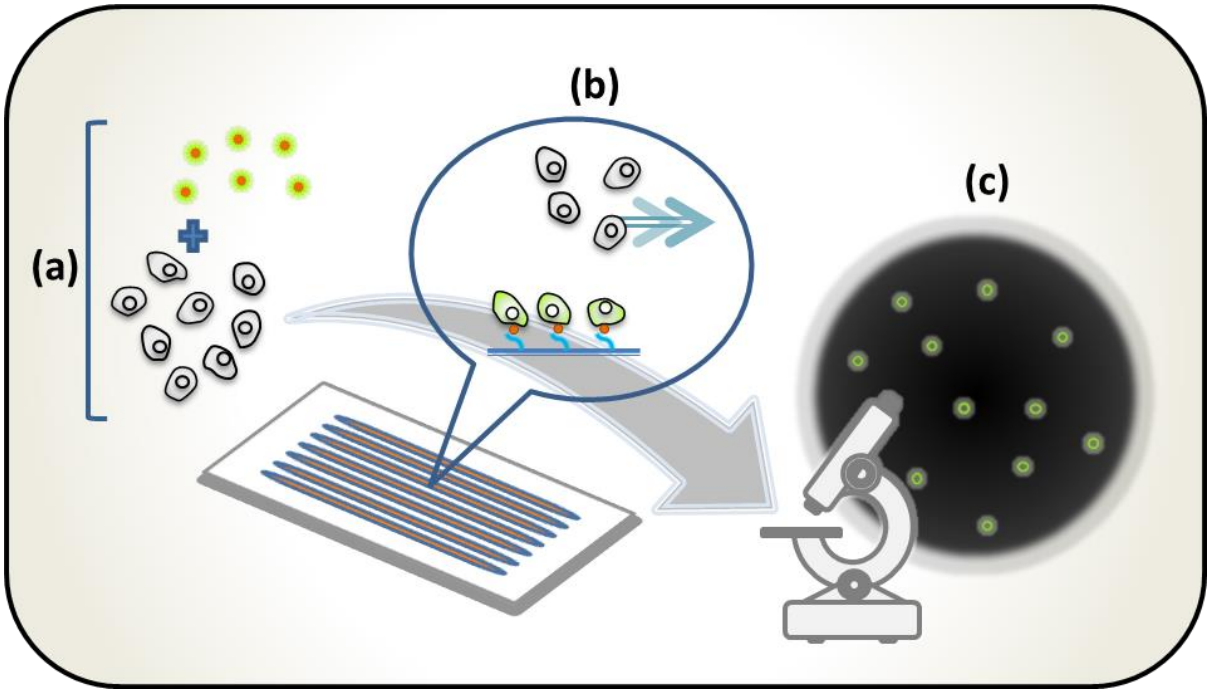

Figure 2. A generalised representation of aptamer-based CTC capture in a microfluidic. a) Cells are incubated with a cell specific fluorescent dye. b) Aptamers specifically capture CTCs whilst other cells are removed from the microfluidic in the flowing buffer. c) Cells captured in the microfluidic are observed, measured and counted by fluorescence microscopy.

3.3 Aptamer based microfluidic protein sensing

Given the advantages of aptamer based microfluidics, emerging platforms for protein sensing are being developed for diagnostics. Here we classify into three types: 1/ direct detection, 2/ sandwich assay and 3/ label-free detection.

3.3.1 Direct detection

One of the major characteristics of aptamers is the ease by which they can be chemically modified. There are well-established protocols for nucleic acid modification for labelling nucleic acids with different tags to facilitate direct detection.[75] Figure 3A illustrates the general configuration of direct detection approach by using fluorescently labelled aptamers.

George Whitesides discussed the crosstalk between CE and microfluidic analytical methods.[17a] Besides aptamer selection, CE can also be applied for aptamer-based protein sensing. The first aptamer based CE method for protein sensing was reported by Kennedy and colleagues in 1998.[76] They demonstrated a method by integrating two approaches of CE, affinity probe capillary electrophoresis (APCE) and capillary electrophoresis with laser-induced fluorescence detection (CE-LIF), for the detection of immunoglobulin E (IgE). APCE is based on the separation of the target molecule bound to a fluorescence-labelled affinity probe in a mixture by using CE. In Kennedy and colleagues' study, IgE was bound to a fluorophore-labelled aptamer and separated by CE. The signal of the IgE-fluorophore labelled aptamer complex was subsequently detected by laser induced fluorescence.[76] Based on this approach, they achieved a limit of detection (LoD) of 46 pM and detected thrombin with an LoD of 40 nM.

Recent advances in electrochemical sensing also benefit direct detection of proteins in microfluidics. Liu *et al.* described a microdevice that consists of microfluidic channels and aptamer-modified electrodes for detecting cytokines, interferon (INF)-γ and tumour necrosis factor (TNF)-α, released from leukocytes.[77] Thiolated aptamers were functionalised by using the redox reporter methylene blue and immobilised to gold electrodes. Binding to the cytokines induced a conformational change in the aptamers, shifting the redox active methylene blue group resulting in a signal-off reading by square wave voltammetry due to a decrease in current.

3.3.2 Sandwich assay

The high specificity of aptamers not only enables them to act as signal reporters in microfluidic systems, it also facilitates the specific targeting of proteins for further detection in sandwich assays (Figure 3B). A method to detect interleukin-8 (IL-8) that incorporates rolling circle amplification (RCA) was developed by Zhang and colleagues.[78] Using a dual function bio-

chip, endothelial cells were grown in a cell culture chamber connected to a detection chamber by micro-channels. The detection chamber was coated with anti-IL-8 antibodies for capturing the secreted IL-8. The presence of IL-8 then was detected by using a biotinylated aptamer which bound to avidin. The presence of avidin enables the binding of biotinylated RCA primers for further signal amplification by RCA. The LoD of this RCA incorporated sandwich assay for detection of IL-8 was 7.5 pg/mL.

Instead of coating aptamers on the surface of microfluidic channels, aptamers can also be immobilised to microbeads for capturing target proteins. Conventional approaches for detecting the cardiovascular disease biomarker, C-reactive protein (CRP), take 150 minutes and the detection limit is 0.125mg/L.[79] Yang and colleagues developed an automatic microfluidic process for measuring C-reactive protein (CRP) by a magnetic bead-incorporated microfluidic system, which significantly improved the efficiency and LoD.[79] The advantage of utilising magnetic beads in microfluidic system is related to their facile and fast separation by magnetic field. Thus, targets captured on the surface of aptamer immobilised magnetic beads can be efficiently isolated. In the Yang *et al.* system, aptamers were immobilised on magnetic beads for capturing CRP, and the whole process is driven by micropumps, microvalves and micromixers for washing and separation of unbound species. After washing steps, the captured CRP was detected by antibodies for further chemiluminescence measurements. With this automatic microfluidic system, lower quantities of sample and reagents were required, process time was significantly reduced to 25 minutes, and the detection limit was greatly improved to 0.0125mg/L.

3.3.3 Label-free detection

Label-free detection is another aspect of aptamer-based microfluidic detection (Figure 3C). A *Plasmodium falciparum* lactate dehydrogenase (PfLDH) specific aptamer was identified by

our team (Tanner research group) [80] and an aptamer-tethered enzyme capture (APTEC) assay which bases detection on the intrinsic enzyme activity of the captured PfLDH malaria biomarker protein was developed [81]. It has since been incorporated to a range different detection formats.[9d, 9e, 82] The APTEC assay was incorporated into a portable microfluidic 3D printed device as a prototype point-of-care diagnostic device [82a]. The PfLDH-specific aptamer was immobilised on the surface of magnetic beads. The aptamer beads within the microfluidic were incubated in patient blood samples, separated from the blood, washed, and brought into the development chamber. If bound blood-borne PfLDH was present on the beads, then the intrinsic enzyme activity of PfLDH in the development chamber resulted in the generation of a colored diformazan dye which was detected by a mobile detection system. The performance of this 3-D printed microfluidic device was examined by using both cultured parasites samples and clinical samples and found it able to differentiate *P. falciparum* infected and uninfected samples with lower detection limit of 0.01 % parasitaemia.[82a]

Colorimetric detection has also been developed for aptamer-based microfluidic protein biosensing. The advantage of using colorimetric detection is that the signal can be observed by the naked eye without specialised instrumentation, which favours protein sensing in closed systems. A G-quadruplex DNAzyme which consists of a G-rich nucleic acid sequences that forms non-canonical hemin associating structures of stacked G-tetrads shows peroxidase-mimicking activity. [83] These DNAzyme structures are able to react with peroxidase substrates to produce colorimetric signals. By incorporating G-quadruplex DNAzyme sequences with aptamers, the presence of aptamer captured target can be detected. An aptamer-based microfluidic system with dual chambers was developed for studying cell-to-cell communication between tumour cells and endothelial cells.[83] To demonstrate cell-cell interaction, the online analysis of cell secretory protein, vascular endothelial growth factor (VEGF), was detected by DNAzyme functionalised aptamer that was coated on a microfluidic

channel. [83] On target capture the VEGF aptamer capture induced formation of the peroxidase DNAzyme. [83] By adding hemin and chromogenic peroxidase substrate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), the aptamer captured VEGF was colorimetrically observed.

Electrochemical sensing has also been applied to protein biosensing in the form of label free detection. Electrochemical impedance spectroscopy (EIS) has been employed for the measurements of molecular interactions. [84] EIS measurement is mainly based on the electron transfer kinetics between a redox probe and an electrode. [84] The presence of target can be detected by comparing the circuit resistance between the working electrode and reference electrode. The EIS approach provides high sensitivity with relative simplicity, thus it is widely used in biosensing. Electrochemical approaches for thrombin detection have been developed,[84] and recently it was incorporated to polydimethylsiloxane (PDMS) channel layer to form microfluidic detection.[85] In the microfluidic electrochemical thrombin aptasensor, thrombin binding aptamer was immobilised on a gold working electrode and the presence of thrombin was detected by measuring change in impedance once the binding event occurs. This thrombin microfluidic aptasensor had a detection range of 0.1-100,000 ng/mL and a limit of detection of 0.1 ng/mL. Moreover, compared with traditional electrochemical sensors, this microfluidic aptasensor used less reagent and is thus more cost-efficient.

Microfluidic aptamer-based electrochemical biosensing system has also been demonstrated for the cardiac damage biomarker, creatine kinase (CK)-MB. [86] Only trace amount of CK-MB is secreted when there is cardiac damage. The current gold standard detection method is based on an enzyme-linked immunosorbent assay (ELISA) that requires a substantial sample volume and has low sensitivity. [86] By exploiting the advantages of microfluidics, Shin and colleagues developed an aptamer-based electrochemical biosensor integrated with a microfluidic platform

for in-line detection of secreted CK-MB.[86] This provided a sensitive, low sample approach for the detection of CK-MB.

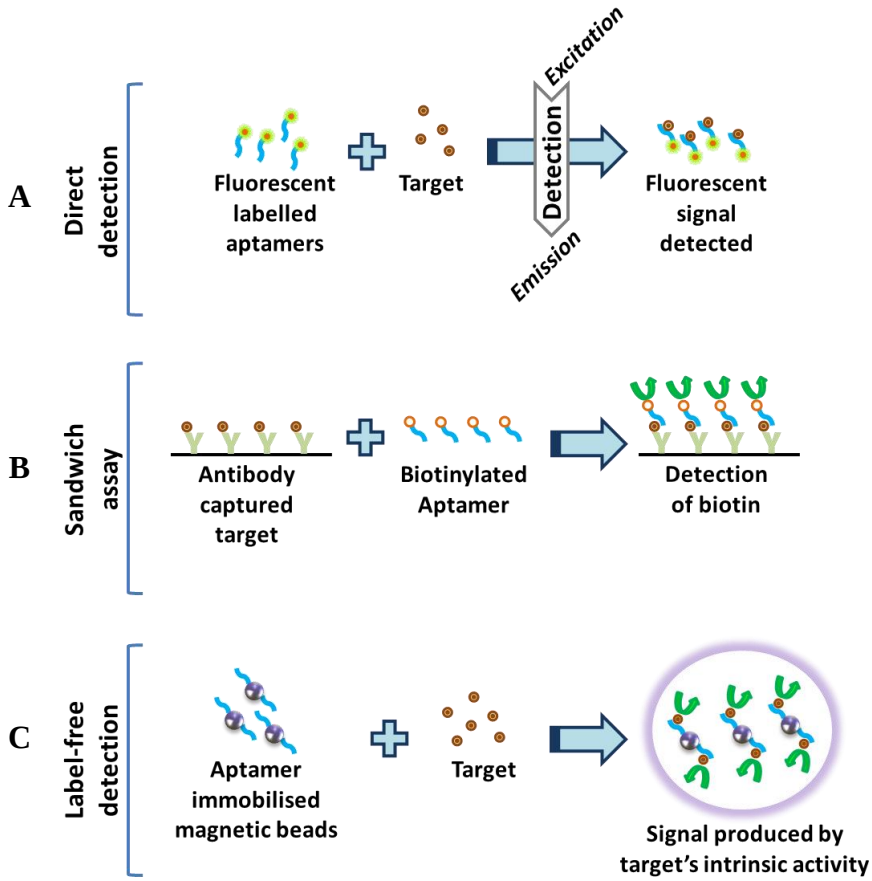

Figure 3. Schematic illustration of different aptamer based detection approaches. A) Aptamer is labelled with reporter for direct detection. B) A sandwich is formed by an antibody for capturing the target, target molecule and a biotin labelled aptamer for further visualisation. C) An example for label-free detection. The magnetic beads act as the solid support for the aptamer for capturing protein targets in sample solution. Colorimetric signal produced by the intrinsic enzyme activity of the target indicating the presence of target.

3.4 Aptamer based microfluidic small molecule detection

Adenosine and ATP are among the most thoroughly researched small molecules in microfluidic biosensor development due to their relevance in various biological reactions and their associations with disease.[87] Commonly used detection approaches include fluorescence,

colorimetry, and electrochemistry. All these approaches have been applied successfully in independent microfluidic systems.[88] Zhang *et al*. applied multienzyme-linked nanoparticle amplification and quantum dots labelling to achieve remarkable 0.1 pM LoD of adenosine using a fluorescence based method.[88a] In terms of colorimetric detection, many groups have developed paper-based microfluidic systems (μPADs) to realize the Point-of-Care Testing (POCT) of adenosine. Tian and colleagues introduced hydrogel-μPADs that reached 15.7 μM detection limit,[88b] while Zhang Group created strip-like μPADs allowing LoD of 0.16 μM.[88c] Another facile method known as Rubik's Cube Stamping (RCS) was also utilized to fabricate a rosin-patterned eight-channel μPAD facilitating adenosine detection as low as 5.7 μM.[88d] Additionally, chemiluminescence based alternative enabled detection of 6 μM of adenosine from 2 μL sample.[89] In contrast to adenosine, ATP detection generally relies on electrochemical methods. Du *et al*. coupled an electrochemical probe named RuHex used signal amplifying AuNPs to obtain 0.3 nM LoD for ATP.[88e] Methods such as electroluminescence (ECL) and photoelectrochemistry (PEC) were have also been explored. The Huang group generated a novel porous Au-paper working electrode that embedded a microfluidic origami ECL device (μ-OECLD) which allowed impressive ATP detection as low as 0.1 pM. [90] The same group further demonstrated the feasibility of their PEC origami device (μ-PECOD) and achieved a 0.2 pM LoD.[91]

Moreover, cocaine, various food contaminant toxin targets such as aflatoxin,[93] botulinum toxin,[94] and ochratoxin A have all gained the attention from aptamer based microfluidic researchers.[95] The Pavesi Group developed a microring resonator to detect aflatoxin in the nanomolar range.[93b] Surface enhanced Raman spectrometry (SERS) was also introduced in a microfluidic system for the detection of ochratoxin A and polychlorinated biphenyls. [96] Due to surface-enhanced effects from patterned metallic nanostructures in microfluidics, the Raman signals of aptameric monolayer assemblies can be improved by several orders of magnitude

(typically 10^6–10^8 enhancement) as compared with standard Raman measurements.[95c, 96] Additionally, Kim *et al*. established a static light scattering detection method in a Y- channel PDMS microfluidic device against oxytetracycline antibiotic, achieving a striking detection limit of 100 ppb with a detection time of less than three minutes.[97] Other small molecules, such as beta-hydroxybutyrate,[98] catecholamine,[99] cortisol,[100] sulforhodamine B,[101] and various types of antibiotics were also used as detection targets in microfluidic systems.[102]

Microfluidic Electrochemical Detector for *In vivo* Continuous monitoring (MEDIC) was introduced by Soh and Plaxco Groups to observe the concentration dynamics of small molecules, particularly doxorubicin (a chemotherapeutic drug) and kanamycin (an antibiotic)[16e]. They were able to track the chemicals in live rats over multiple hours in real-time and envisioned that this system could be applied to human patients.[16e] They further improved the system by utilizing generalizable closed-loop approach to both monitor doxorubicin continuously and adjust the concentration of the drug in live rabbits.[103] Other than small molecules, detection of some metal ions through microfluidic systems has also been explored.[104] Mercury ions could be detected down to 5 ppb through a fluorescent lateral flow aptamer assay in an integrated smartphone-based portable device for point-of-care detection.[95b] DNA aptamers for metal sensing have been suggested to be more widely integrated in microfluidic biosensors.[105]

Table 1. Examples of aptamer-based microfluidics as biosensing tools.

Category	Target	Efficiency [#]	Detection Method
Whole Cell	T-cell acute lymphocytic leukaemia cell line	>80% Efficiency [69a, 106]	Fluorescent [69a, 106]
	B-cell, human Burkitt's lymphoma	61% Efficiency	Fluorescent [69a]
	non-Hodgkin's B-cell lymphoma	50% Efficiency	Fluorescent [69a]
	Lymph Node Carcinoma of the Prostate Cells	90% Recovery	Electrochemical [67]
	Colorectal adenocarcinoma	97% Efficiency	Fluorescent [65]
	Dukes' type C Colorectal carcinoma	91% Efficiency	Fluorescent [65]
	Non-small-cell lung cancer	>95% Efficiency	Fluorescent [107]
	Adenocarcinoma cells	>75% Efficiency	Fluorescent [60]
	H460 large cell carcinoma	>50% Efficiency	Fluorescent [60]
	Pulmonary mucoepidermoid carcinoma cells	>50% Efficiency	Fluorescent [60]
	H1299 large cell carcinoma	>50% Efficiency	Fluorescent [60]
	Squamous carcinoma cells	>50% Efficiency	Fluorescent [60]
	Michigan Cancer Foundation-7 cells	94% Efficiency [108]	Fluorescent [108]
Proteins	Immunoglobulin E	LoD: 46 pM	Fluorescent [76]
	Thrombin	LoD: 40 nM	Fluorescent [76]
		LoD: 0.1 ng/mL	Electrochemical [85]
	Interferon interferon (INF)-γ	LoD: 60 pM	Electrochemical [77b]
	Tumour necrosis factor (TNF)-α	LoD: 0.58 nM	Electrochemical [77a]
	Interleukin-8 (IL-8)	LoD: 7.5 pg/mL	Fluorescent [78]
	C-reactive protein (CRP)	LoD: 0.0125mg/L	Chemiluminescent [79]
	P. falciparum lactate dehydrogenase (PfLDH)	LDL: 0.01% parasitaemia	Colorimetric [82a]
	Vascular endothelial growth factor (VEGF)	LoD: 1.30 ng/mL	Colorimetric [83]
	Creatine Kinase-MB (CK-MB)	LoD: 2.4pg/mL	Electrochemical [86]
Small Molecules	Adenosine	LoD: 0.1 pM	Fluorescent [88a]
		LDL: 15.7 μM[109], 100 μM [88b], LoD: 0.16 μM [88c], 5.7 μM [88d]	Colorimetric [88b-d]
		LDL: 6 μM	Chemiluminescent [89]
	Aflatoxin B1	LoD: 1.77 nM	Colorimetric [93a]
	Aflatoxin	LoD: 1.6×10^{-6} RIU	Ring resonation [93b]
	Ampicillin	LDL: 100 pM	Electrochemical [102a]
	ATP	LoD: 0.3 nM	Electrochemical [88e]
		LDL: 0.1 pM	Electroluminescent [91]
		LoD: 0.2 pM	Photoelectrochemical [91]
	Beta-hydroxybutyrate	LoD: 0.3 mM	Electrochemical [98]
	Botulinum toxin	LDL: 1 pg/ μL	Electrochemical [94]
	Catecholamine	LDL: 50 μM	Electrochemical [99]
	Cocaine	LoD: 4.5 μM [109], 3.8 μM [88b], 2.36 μg[92a]	Colorimetric [88b, 92a]
		LoD: 0.5 pM [88a], 10 pM [92b], 0.2 μM[92c]	Fluorescent [88a, 92b, 92c]
		LoD: 70 nM [88e], LDL: 10 μM [92d]	Electrochemical [88e, 92d]
	Cortisol	LDL: 30 pg/ mL	Electrochemical [100]
	Doxorubicin	LoD: 10 nM in buffer [16e], 60 nM in blood [103]	Electrochemical [16e, 103]
	Kanamycin	LDL: 4.5 mM [16e], 10 nM [102a]	Electrochemical [16e, 102a]
		LoD: 0.29 pg/ mL	Fluorescent [102b]
	Ochratoxin A	LDL: 1.27 nM	Colorimetric [95a]
		LoD: 3 ng/ mL	Fluorescent [95b]
		LDL: 2.5 μM	SERS [95c]
	Oxytetracycline	LDL: 100 ppb	Static Light Scattering [97]

	Polychlorinated biphenyls	LoD: 10 nM	SERS [96]
	Sulforhodamine B	LDL: 0.07 μM	Fluorescent [101]
	Tetracycline	LDL: 1 μM	Fluorescent [102c]

[#]LoD – Limit of detection; LDL – Lower detection limit

3.5 Nanostructures in Microfluidic Sensing.

Given the nature of aptamers as nucleic acids, they can be easily integrated into nucleic acid-based nanostructures for applications in diagnostics, therapeutics and even bioelectronics. The field of DNA nanotechnology originated from Nadrian Seeman's work on using the concept of Holliday junctions to fabricate different two and three-dimensional lattices. [110] Since then, more complicated objects have been created in the form of DNA origami. DNA origami consists of single-stranded plasmid such as M13 used as a scaffold strand, bent into any shapes using sequence specific staple strands.[111] The biocompatibility of DNA has spurred research into nanostructures for medical applications. In terms of diagnostics, simple DNA nanostructures have been developed to detect nucleic acids target through strand displacement. The presence of target induces the changes in distance between the parts of nanostructure, which leads to the generation of signal.[112] For detecting molecules other than nucleic acids, the target recognition site of nanostructure can be replaced with target specific aptamers. Depending on the design of structural changing mechanism, a split aptamer could be essential in bringing parts of the structure into proximity for signal generation.[9e]

The programmability of nucleic acids allows for the integration of different DNA nanostructures to improve the performance of aptamer-based microfluidic systems. The Soh group previously published the work on using microfluidics system to analyze and select the shape-changing DNA nanostructure for cargo release upon target recognition.[113] The typical systematic evolution of ligands by exponential enrichment (SELEX) selects single stranded nucleic acids for specific target binding.[1b] Instead of doing it in the traditional way, they use the same concept to directly select a split aptamer that could eliminate the issue of reduction in affinity when modifying a single-stranded aptamer into a split. The two random regions of split aptamer were incorporated to the ends of a GC-rich duplex so that the guanine rich sequence was able to form a G-quadruplex and the duplex was intercalated with a fluorescent molecule as a cargo. The strong binding between split aptamer and target molecule would displace the

complementary strand in the duplex leading to the formation of G-quadruplex and release the fluorescent molecule with enhanced fluorescence intensity.[113] By using micromagnetic separation, they could isolate the complementary strand that was conjugated to streptavidin bead and measure the fluorescence signal on individual beads in the microfluidic chip.

The attachment of aptamers to the vertex of DNA tetrahedron nanostructures has been found to enhance the binding affinity of aptamers towards target molecules. The Pei group attempted to coat three different DNA tetrahedron to the inner surface of a microfluidic channel capturing three different analytes including ATP, thrombin and cocaine as shown in Figure 4A.[114] Multiplex detection was achieved in blood serum and the biomarkers were detected within the clinical range. In addition to the reduction in cost of analysis due to the use of nanoliter droplets, detection of biomarkers in serum indicates a synergy between DNA nanostructures and microfluidics for disease diagnosis. [114] Apart from enhancing sensitivity of detection, the sophisticated design of microfluidics chip also allows development of molecular computing approaches. Most designs of structural switching DNA nanostructures use a "burnt-bridge" mechanism, an irreversible step.[115] With multiple inputs and computer-controlled flow rate, researchers created 64 commands to control a bipedal DNA walker to move bi-directionally on a DNA origami track with defined speed (Figure 4B – E).[116] It was achieved by introducing fuel and antifuel strands separately in order to perform multiple strand-displacement, an example of an advanced nanostructure techniques that could have applications in aptamer based biosensing.

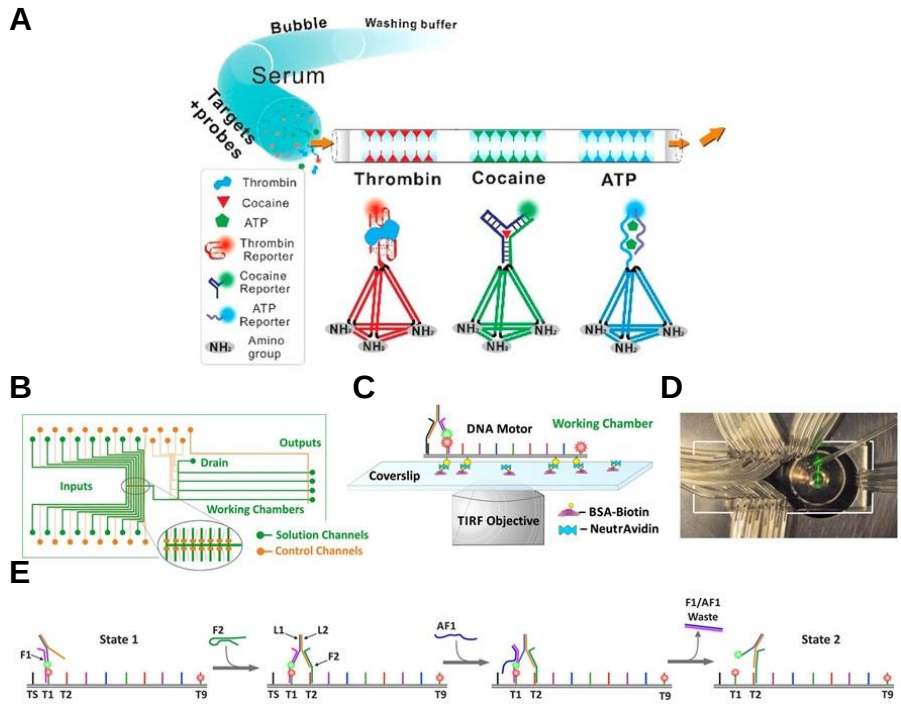

Figure 4. Integration of DNA nanostructure to microfluidics systems. A) Design of multiplex sensing using DNA tetrahedra with aptamers in microfluidic channels. Tetrahedra with amino groups extend from the bottom were coated on the inner surface of glass capillary through amine-aldehyde reaction. Different aptamers extended from the top of tetrahedra allowed simultaneous detection of thrombin, cocaine and ATP in a nanoliter droplet. B) Microfluidic device with sixteen input channels (green) with pneumatic valves (orange). The combinations allowed 64 consecutive commands of DNA strands, leading to 32 steps of a bipedal walker on a DNA origami track inside the microfluidics. C) The DNA origami walker system was immobilized on the coverslip through biotin-NeutrAvidin interaction. D) Image of the microfluidic device mounted on the TIRF setup. E) Mechanism of the walker system on DNA origami. Fuel strands (F) targeted to specific legs of walker and foothold (T) on origami would lead to precise immobilization. Antifuel strands (AF) were used to displace the fuel strands for leg detachment as completing one movement. The increasing distance between leg and foothold was monitored by the change in fluorescence resonance energy transfer (FRET) signal. Adapted with permission from Qu *et al.*,[114] and Tomov *et al.*[116].

In addition to DNA nanostructures, there are also nanomaterials in microfluidics that enhance the sensing performance of aptamers. The first example of such integration was performed by Langer's group in 2005 [117]. They conjugated aptamer targeting transmembrane prostate specific membrane antigen (PSMA) to PEGylated poly(lactic acid) nanoparticles. The nano-conjugate could adhere to cells seeded in microchannel and resist at least 15 times higher shear force than the nanoparticle without aptamer. This additional barrier of shear force enhanced the specificity of the detection. Since PSMA is a cell surface epitope of prostate cancer, the same group also demonstrated the use of this nanoparticle to encapsulate cisplatin and docetaxel for target drug delivery.[118] The nanoparticle was synthesized by nanoprecipitation using a microfluidic device that controlled the flow rate of polymers and drug hence the size of nanoparticle. Simplifying the design of such microfluidic devices for more practical applications at a lower cost is important. In 2012, a three-dimensional microfluidic system was built on a paper-folding origami for point of care diagnosis. [90] The paper surface was modified with porous gold nanoparticle for generation of an electrochemiluminescence signal. The incorporation of an ATP aptamer to the nanoparticle led to the detection limit of 0.1 pM. A similar concept was applied to another paper microfluidic device (μPAD) as a colorimetric assay to detect cocaine and kanamycin with the limit of detection of 7.78 nM and 3.35 nM respectively.[92a] A platinum nanoparticle functionalized carbon nanotube for the detection of mercury ion as an indication of environmental contamination also demonstrated a detection limit of 56 pM.[119]

Single microfluidic devices allow multiplexed detection for cost-effective diagnosis.[88a, 120] In one study gold nanorods were conjugated to three aptamers; thrombin-binding aptamer, immunoglobulin E binding aptamer and PSMA.[120a] The aptamer modified gold nanorods were immobilized into channels on a microfluidic chip then the antigen sample was injected along the main channel. Detected target led to a proportional response in localized surface plasmonic

resonance that could be detected by dark-field microscopy. Different designs of microfluidic chip were also found to detect multiple foodborne pathogens with colorimetric signal in food samples.[120b] The detection of *Salmonella enterica*, *Escherichia coli*, and *Listeria monocytogenes* reached the limit of detection of 10 colony-forming unit (CFU) per mL. All these studies show the versatility of using DNA nanostructures and microfluidics for both biomedical and bioelectronic applications.

Our group previously developed three DNA nanostructures with different mechanisms for the detection of malaria biomarker *Plasmodium falciparum* lactate dehydrogenase (PfLDH). [9d-f] First, we were able to use a protein target to open the lid of a DNA origami box through strand displacement.[9d] We subsequently developed a split aptamer which facilitates a closing mechanism for DNA nanotweezers.[9e] The presence of PfLDH closed the tweezers and led to the formation of a complete G-quadruplex which catalyzed a peroxidase reaction which generated a diagnostic signal.[9d, 9e] Large quantities of reagents were required to compare the performance of nanostructures during the optimization process. Microfluidic technology could allow for a more precise optimization process and therefore provide a reduction in the development costs. For diagnostic purposes, we also studied the efficiency of various DNA polyhedra which enhanced the sensitivity of PfLDH aptamer.[9f] The elevation of the aptamer from the coated surface lowered the dissociation constant six-fold. This enhancement could potentially eliminate false-negative results providing a more reliable diagnosis.[82a] Together with the integration in microfluidics such as the one made by the Pei group,[114] we can foresee the development nanoscale biosensors for preventive diagnosis with much higher sensitivity and reliability.

4. Conclusion and Future Perspectives

Microfluidic technologies have thoroughly enhanced aptamer-based technologies, from aptamer selections to aptamer-based biosensors. One of the earliest microfluidic methods to benefit aptamer researchers was capillary electrophoresis, a microfluidic method that has been

further improved with droplet microfluidics and has been used to discover an array of important aptamers. Micromagnetic beads have complemented both microfluidics and aptamer research, first benefiting researchers looking to partition aptamer bound complexes from solution they have been developed into integrated systems that can perform entire rounds of SELEX and has resulted in the discovery of a range of important aptamers. IVC is a method which has taken SELEX to a new paradigm, in which aptamers are selected not only for binding, but also for catalytic activity. Based on droplet microfluidics an array of DNA structures has been developed to catalyse a range of chemical reactions. Aptamer based microfluidics have enabled improvements in biosensing for whole cells, proteins, and small molecules. This is ultimately highlighted by implantable microfluidic devices that can measure the concentration of biomarkers in the whole blood of live rats. Despite all the successes, it is clear that we are still at an early stage of discovery at the interface between microfluidics and aptamer technology.

Microfluidic aptamer selection has many possible application and research directions. IVC has been utilized for the selection of nucleic acid aptamers to expand selection type from the simple binding and single turnover catalysis of classical SELEX, to the more exotic properties such as turn on fluorescence upon aptamer/target binding and multiple turnover catalysis. Selections can potentially be imagined for any assay that can be linked to a fluorogenic reporter. This opens a huge number of potential of research directions including catalysis, bioprocessing and photonics.

Aptamer based microfluidic systems in general, whether they be for selection or sensing, allow for increasing throughput, decreasing experimental complexity and increasing sensitivity of the assay experiment being performed. These three experimental characteristics are paramount to converting labour-intensive, expensive lab based assays into a format that is suitable for translational applications. There is likely an important future for aptamer-based microfluidic

sensing across disease detection, biosecurity, environmental monitoring and a wide range of other areas.

5. Acknowledgements

This work was supported by Hong Kong University Grants Council under Research Grants Council (RGC) General Research Fund (GRF) grants 17163416 and 17127515 to JAT, and under General Research Fund (GRF) grants 17304514 and 17329516 to HCS.

Received: ((will be filled in by the editorial staff))

Revised: ((will be filled in by the editorial staff))

Published online: ((will be filled in by the editorial staff))

References

[1] aA. D. Ellington, J. W. Szostak, *Nature* **1990**, *346*, 818; bC. Tuerk, L. Gold, *Science* **1990**, *249*, 505.

[2] aD. Shangguan, Y. Li, Z. Tang, Z. C. Cao, H. W. Chen, P. Mallikaratchy, K. Sefah, C. J. Yang, W. Tan, *Proceedings of the National Academy of Sciences* **2006**, *103*, 11838; bG. Mayer, *Angewandte Chemie International Edition* **2009**, *48*, 2672-2689; cM. Mascini, I. Palchetti, S. Tombelli, *Angewandte Chemie International Edition* **2012**, *51*, 1316-1332.

[3] S. D. Jayasena, *Clinical Chemistry* **1999**, *45*, 1628.

[4] aY. Wan, Y.-t. Kim, N. Li, S. K. Cho, R. Bachoo, A. D. Ellington, S. M. Iqbal, *Cancer Research* **2010**, *70*, 9371; bY. Hathout, E. Brody, P. R. Clemens, L. Cripe, R. K. DeLisle, P. Furlong, H. Gordish-Dressman, L. Hache, E. Henricson, E. P. Hoffman, Y. M. Kobayashi, A. Lorts, J. K. Mah, C. McDonald, B. Mehler, S. Nelson, M. Nikrad, B. Singer, F. Steele, D. Sterling, H. L. Sweeney, S. Williams, L. Gold, *Proceedings of the National Academy of Sciences* **2015**, *112*, 7153; cE. S. Gragoudas, A. P. Adamis, E. T. Cunningham, M. Feinsod, D. R. Guyer, *New England Journal of Medicine* **2004**, *351*, 2805-2816.

[5] L. H. Lauridsen, R. N. Veedu, *Nucleic Acid Therapeutics* **2012**, *22*, 371-379.

[6] J. B. H. Tok, N. O. Fischer, *Chemical Communications* **2008**, 1883-1885.

[7] G. Pothoulakis, F. Ceroni, B. Reeve, T. Ellis, *ACS Synthetic Biology* **2014**, *3*, 182-187.

[8] aK. D. Warner, M. C. Chen, W. Song, R. L. Strack, A. Thorn, S. R. Jaffrey, A. R. Ferré-D'Amaré, *Nature Structural &Amp; Molecular Biology* **2014**, *21*, 658; bN. Svensen, Samie R. Jaffrey, *Cell Chemical Biology* **2016**, *23*, 415-425.

[9] aS. M. Douglas, I. Bachelet, G. M. Church, *Science* **2012**, *335*, 831; bS. Li, Q. Jiang, S. Liu, Y. Zhang, Y. Tian, C. Song, J. Wang, Y. Zou, G. J. Anderson, J.-Y. Han, Y. Chang, Y. Liu, C. Zhang, L. Chen, G. Zhou, G. Nie, H. Yan, B. Ding, Y. Zhao, *Nature Biotechnology* **2018**, *36*, 258; cQ. Mei, R. H. Johnson, X. Wei, F. Su, Y. Liu, L. Kelbauskas, S. Lindsay, D. R. Meldrum, H. Yan, *Nano Research* **2013**, *6*, 712-719; dM. S. L. Tang, S. C.-C. Shiu, M. Godonoga, Y.-W. Cheung, S. Liang, R. M. Dirkzwager, A. B. Kinghorn, L. A. Fraser, J. G. Heddle, J. A. Tanner, *Nanomedicine: Nanotechnology, Biology and Medicine* **2018**, *14*, 1161-1168; eS. C.-C. Shiu, Y.-W. Cheung, R. M. Dirkzwager, S. Liang, A. B. Kinghorn, L. A. Fraser, M. S. L. Tang, J. A. Tanner, *Advanced Biosystems* **2017**, *1*, 1600006; fC. S. Shiu, A. L. Fraser, Y. Ding, A. J. Tanner, *Molecules* **2018**, *23*.

[10] A. I. Taylor, V. B. Pinheiro, M. J. Smola, A. S. Morgunov, S. Peak-Chew, C. Cozens, K. M. Weeks, P. Herdewijn, P. Holliger, *Nature* **2014**, *518*, 427.

[11] J. W. Jorgenson, K. D. Lukacs, *Analytical Chemistry* **1981**, *53*, 1298-1302.

[12] X. Lou, J. Qian, Y. Xiao, L. Viel, A. E. Gerdon, E. T. Lagally, P. Atzberger, T. M. Tarasow, A. J. Heeger, H. T. Soh, *Proceedings of the National Academy of Sciences* **2009**, *106*, 2989.

[13] A. Autour, E. Westhof, M. Ryckelynck, *Nucleic Acids Research* **2016**, *44*, 2491-2500.

[14] A. L. Fraser, B. A. Kinghorn, S. M. Tang, Y.-W. Cheung, B. Lim, S. Liang, M. R. Dirkzwager, A. J. Tanner, *Molecules* **2015**, *20*.

[15] J. J. Agresti, B. T. Kelly, A. Jäschke, A. D. Griffiths, *Proceedings of the National Academy of Sciences of the United States of America* **2005**, *102*, 16170.

[16] aL. Huang, S. Bian, Y. Cheng, G. Shi, P. Liu, X. Ye, W. Wang, *Biomicrofluidics* **2017**, *11*, 011501; bM. Selmi, M. H. Gazzah, H. Belmabrouk, *Scientific Reports* **2017**, *7*, 5721; cY.-J. Liu, S.-S. Guo, Z.-L. Zhang, W.-H. Huang, D. Baigl, M. Xie, Y. Chen, D.-W. Pang, *ELECTROPHORESIS* **2007**, *28*, 4713-4722; dJ. P. Brody, P. Yager, *Sensors and Actuators A: Physical* **1997**, *58*, 13-18; eB. S. Ferguson, D. A. Hoggarth, D. Maliniak, K. Ploense, R. J. White, N. Woodward, K. Hsieh, A. J. Bonham, M. Eisenstein, T. E. Kippin, K. W. Plaxco, H. T. Soh, *Science Translational Medicine* **2013**, *5*, 213ra165; fV. Thiviyanathan, D. G. Gorenstein, *PROTEOMICS – Clinical Applications* **2012**, *6*, 563-573.

[17] aG. M. Whitesides, *Nature* **2006**, *442*, 368; bL. Y. Yeo, H.-C. Chang, P. P. Y. Chan, J. R. Friend, *Small* **2011**, *7*, 12-48.

[18] T. M. Squires, S. R. Quake, *Reviews of Modern Physics* **2005**, *77*, 977-1026.

[19] S. K. Sia, G. M. Whitesides, *Electrophoresis* **2003**, *24*, 3563-3576.

[20] B. H. Weigl, P. Yager, *Science* **1999**, *283*, 346-347.

[21] C.-Y. Lee, C.-L. Chang, Y.-N. Wang, L.-M. Fu, *International Journal of Molecular Sciences* **2011**, *12*.

[22] L. Shang, Y. Cheng, Y. Zhao, *Chemical Reviews* **2017**, *117*, 7964-8040.

[23] J. Nilsson, M. Evander, B. Hammarström, T. Laurell, *Analytica Chimica Acta* **2009**, *649*, 141-157.

[24] J. Kim, M. Johnson, P. Hill, B. K. Gale, *Integrative Biology* **2009**, *1*, 574-586.

[25] C.-C. Hong, J.-W. Choi, C. H. Ahn, *Lab on a Chip* **2004**, *4*, 109-113.

[26] C. Wyatt Shields Iv, C. D. Reyes, G. P. López, *Lab on a Chip* **2015**, *15*, 1230-1249.

[27] F. Lan, B. Demaree, N. Ahmed, A. R. Abate, *Nature Biotechnology* **2017**, *35*, 640.

[28] V. Lecault, M. VanInsberghe, S. Sekulovic, D. J. H. F. Knapp, S. Wohrer, W. Bowden, F. Viel, T. McLaughlin, A. Jarandehei, M. Miller, D. Falconnet, A. K. White, D. G.

Kent, M. R. Copley, F. Taghipour, C. J. Eaves, R. K. Humphries, J. M. Piret, C. L. Hansen, *Nature Methods* **2011**, *8*, 581.
[29] J. W. Parks, M. A. Olson, J. Kim, D. Ozcelik, H. Cai, R. Carrion, J. L. Patterson, R. A. Mathies, A. R. Hawkins, H. Schmidt, *Biomicrofluidics* **2014**, *8*, 054111.
[30] G. Luka, A. Ahmadi, H. Najjaran, E. Alocilja, M. DeRosa, K. Wolthers, A. Malki, H. Aziz, A. Althani, M. Hoorfar, *Sensors* **2015**, *15*.
[31] S. Sakuma, F. J. J. o. R. Arai, *Journal of Robotics and Mechatronics* **2013**, *25*, 277-284.
[32] S. M. Lunte, D. M. Radzik, in *Progress in Pharmaceutical and Biomedical Analysis, Vol. 2* (Eds.: S. M. Lunte, D. M. Radzik), Elsevier, **1996**, pp. 3-21.
[33] S. D. Mendonsa, M. T. Bowser, *Journal of the American Chemical Society* **2004**, *126*, 20-21.
[34] M. Berezovski, A. Drabovich, S. M. Krylova, M. Musheev, V. Okhonin, A. Petrov, S. N. Krylov, *Journal of the American Chemical Society* **2005**, *127*, 3165-3171.
[35] M. V. Berezovski, M. U. Musheev, A. P. Drabovich, J. V. Jitkova, S. N. Krylov, *Nature Protocols* **2006**, *1*, 1359.
[36] S. Javaherian, M. U. Musheev, M. Kanoatov, M. V. Berezovski, S. N. Krylov, *Nucleic Acids Research* **2009**, *37*, e62-e62.
[37] J. Tok, J. Lai, T. Leung, S. F. Y. Li, *ELECTROPHORESIS* **2010**, *31*, 2055-2062.
[38] aJ. Ashley, K. Ji, S. F. Y. Li, **2012**, *33*, 2783-2789; bS. Lisi, E. Fiore, S. Scarano, E. Pascale, Y. Boehman, F. Ducongé, S. Chierici, M. Minunni, E. Peyrin, C. Ravelet, *Analytica Chimica Acta* **2018**, *1038*, 173-181.
[39] R. Yufa, S. M. Krylova, C. Bruce, E. A. Bagg, C. J. Schofield, S. N. Krylov, *Analytical Chemistry* **2015**, *87*, 1411-1419.
[40] J. G. Bruno, *Biochemical and Biophysical Research Communications* **1997**, *234*, 117-120.
[41] A. S. Sadeghi, M. Mohsenzadeh, K. Abnous, S. M. Taghdisi, M. Ramezani, *Talanta* **2018**, *182*, 193-201.
[42] J. Qian, X. Lou, Y. Zhang, Y. Xiao, H. T. Soh, *Analytical Chemistry* **2009**, *81*, 5490-5495.
[43] aM. Cho, Y. Xiao, J. Nie, R. Stewart, A. T. Csordas, S. S. Oh, J. A. Thomson, H. T. Soh, *Proceedings of the National Academy of Sciences* **2010**, *107*, 15373; bS. S. Oh, K. M. Ahmad, M. Cho, S. Kim, Y. Xiao, H. T. Soh, *Analytical Chemistry* **2011**, *83*, 6883-6889.
[44] Q. Wang, W. Liu, Y. Xing, X. Yang, K. Wang, R. Jiang, P. Wang, Q. Zhao, *Analytical Chemistry* **2014**, *86*, 6572-6579.
[45] G. Hybarger, J. Bynum, R. F. Williams, J. J. Valdes, J. P. Chambers, *Analytical and Bioanalytical Chemistry* **2006**, *384*, 191-198.
[46] C.-J. Huang, H.-I. Lin, S.-C. Shiesh, G.-B. Lee, *Biosensors and Bioelectronics* **2010**, *25*, 1761-1766.
[47] C.-J. Huang, H.-I. Lin, S.-C. Shiesh, G.-B. Lee, *Biosensors and Bioelectronics* **2012**, *35*, 50-55.
[48] H.-I. Lin, C.-C. Wu, C.-H. Yang, K.-W. Chang, G.-B. Lee, S.-C. Shiesh, *Lab on a Chip* **2015**, *15*, 486-494.
[49] J. Kim, T. R. Olsen, J. Zhu, J. P. Hilton, K.-A. Yang, R. Pei, M. N. Stojanovic, Q. Lin, *Scientific Reports* **2016**, *6*, 26139.
[50] S.-L. Hong, Y.-T. Wan, M. Tang, D.-W. Pang, Z.-L. Zhang, *Analytical Chemistry* **2017**, *89*, 6535-6542.
[51] S.-L. Hong, M.-Q. Xiang, M. Tang, D.-W. Pang, Z.-L. Zhang, *Analytical Chemistry* **2019**, *91*, 3367-3373.
[52] aJ. J. Agresti, E. Antipov, A. R. Abate, K. Ahn, A. C. Rowat, J.-C. Baret, M. Marquez, A. M. Klibanov, A. D. Griffiths, D. A. Weitz, *Proceedings of the National Academy of*

Sciences **2010**, *107*, 4004; bA. Fallah-Araghi, J.-C. Baret, M. Ryckelynck, A. D. Griffiths, *Lab on a Chip* **2012**, *12*, 882-891; cM. Ryckelynck, S. Baudrey, C. Rick, A. Marin, F. Coldren, E. Westhof, A. D. Griffiths, *RNA* **2015**, *21*, 458-469.

[53] J. S. Paige, K. Y. Wu, S. R. Jaffrey, *Science* **2011**, *333*, 642.

[54] aR. L. Strack, W. Song, S. R. Jaffrey, *Nature Protocols* **2013**, *9*, 146; bT. A. Rogers, G. E. Andrews, L. Jaeger, W. W. Grabow, *ACS Synthetic Biology* **2015**, *4*, 162-166.

[55] E. V. Dolgosheina, S. C. Y. Jeng, S. S. S. Panchapakesan, R. Cojocaru, P. S. K. Chen, P. D. Wilson, N. Hawkins, P. A. Wiggins, P. J. Unrau, *ACS Chemical Biology* **2014**, *9*, 2412-2420.

[56] A. Autour, S. C. Y. Jeng, A. D. Cawte, A. Abdolahzadeh, A. Galli, S. S. S. Panchapakesan, D. Rueda, M. Ryckelynck, P. J. Unrau, *Nature Communications* **2018**, *9*, 656.

[57] V. Thiviyanathan, D. G. Gorenstein, *Proteomics. Clinical applications* **2012**, *6*, 563-573.

[58] Y. Song, Z. Zhu, Y. An, W. Zhang, H. Zhang, D. Liu, C. Yu, W. Duan, C. J. Yang, *Analytical Chemistry* **2013**, *85*, 4141-4149.

[59] G. S. Zamay, O. S. Kolovskaya, T. N. Zamay, Y. E. Glazyrin, A. V. Krat, O. Zubkova, E. Spivak, M. Wehbe, A. Gargaun, D. Muharemagic, M. Komarova, V. Grigorieva, A. Savchenko, A. A. Modestov, M. V. Berezovski, A. S. Zamay, *Molecular Therapy* **2015**, *23*, 1486-1496.

[60] Z. Libo, T. Chuanhao, X. Li, Z. Zhen, L. Xiaoyan, H. Haixu, C. Si, Z. Wei, H. Mengfei, F. Anna, L. Bing, T. Hsian‐Rong, G. Hongjun, L. Yi, F. Xiaohong, *Small* **2016**, *12*, 1072-1081.

[61] L. M. Millner, M. W. Linder, R. Valdes, *Annals of clinical and laboratory science* **2013**, *43*, 295-304.

[62] J. Smerage, D. Hayes, *British Journal of Cancer* **2006**, *94*, 8.

[63] J. A. Phillips, Y. Xu, Z. Xia, Z. H. Fan, W. Tan, *Analytical Chemistry* **2009**, *81*, 1033-1039.

[64] U. Dharmasiri, S. Balamurugan, A. A. Adams, P. I. Okagbare, A. Obubuafo, S. A. Soper, *ELECTROPHORESIS* **2009**, *30*, 3289-3300.

[65] W. Sheng, T. Chen, R. Kamath, X. Xiong, W. Tan, Z. H. Fan, *Anal Chem* **2012**, *84*, 4199-4206.

[66] L. R. Huang, E. C. Cox, R. H. Austin, J. C. Sturm, *Science* **2004**, *304*, 987-990.

[67] D. Udara, B. Subramanian, A. A. A., O. P. I., O. Annie, S. S. A., *ELECTROPHORESIS* **2009**, *30*, 3289-3300.

[68] S. J. Reinholt, H. G. Craighead, *Anal Chem* **2018**, *90*, 2601-2608.

[69] aY. Xu, J. A. Phillips, J. Yan, Q. Li, Z. H. Fan, W. Tan, *Analytical Chemistry* **2009**, *81*, 7436-7442; bW. Sheng, T. Chen, R. Kamath, X. Xiong, W. Tan, Z. H. Fan, *Analytical Chemistry* **2012**, *84*, 4199-4206.

[70] L. Wang, Q. I. N. Zheng, Q. A. Zhang, H. Xu, J. Tong, C. Zhu, Y. Wan, *Oncology Letters* **2012**, *4*, 935-940.

[71] Y. Song, Y. Shi, M. Huang, W. Wang, Y. Wang, J. Cheng, Z. Lei, Z. Zhu, C. Yang, *Angewandte Chemie International Edition* **2019**, *58*, 2236-2240.

[72] J. Teng, F. Yuan, Y. Ye, L. Zheng, L. Yao, F. Xue, W. Chen, B. Li, *Frontiers in microbiology* **2016**, *7*, 1426-1426.

[73] J. Chung, J. S. Kang, J. S. Jurng, J. H. Jung, B. C. Kim, *Biosensors and Bioelectronics* **2015**, *67*, 303-308.

[74] Z. Altintas, M. Akgun, G. Kokturk, Y. Uludag, *Biosensors and Bioelectronics* **2018**, *100*, 541-548.

[75] aD. Proudnikov, A. Mirzabekov, *Nucleic acids research* **1996**, *24*, 4535-4542; bP. R. Langer, A. A. Waldrop, D. C. Ward, *Proceedings of the National Academy of Sciences* **1981**, *78*, 6633-6637; cA. Roget, H. Bazin, R. Teoule, *Nucleic acids research* **1989**, *17*, 7643-7651; dJ. Goodchild, *Bioconjugate Chemistry* **1990**, *1*, 165-187.

[76] I. German, D. D. Buchanan, R. T. Kennedy, *Analytical Chemistry* **1998**, *70*, 4540-4545.

[77] aY. Liu, T. Kwa, A. Revzin, *Biomaterials* **2012**, *33*, 7347-7355; bY. Liu, J. Yan, M. C. Howland, T. Kwa, A. Revzin, *Analytical Chemistry* **2011**, *83*, 8286-8292.

[78] W. Zhang, Z. He, L. Yi, S. Mao, H. Li, J.-M. Lin, *Biosensors and Bioelectronics* **2018**, *102*, 652-660.

[79] Y.-N. Yang, H.-I. Lin, J.-H. Wang, S.-C. Shiesh, G.-B. Lee, *Biosensors and Bioelectronics* **2009**, *24*, 3091-3096.

[80] aY.-W. Cheung, R. M. Dirkzwager, W.-C. Wong, J. Cardoso, J. D'Arc Neves Costa, J. A. Tanner, *Biochimie* **2018**, *145*, 131-136; bY.-W. Cheung, J. Kwok, A. W. L. Law, R. M. Watt, M. Kotaka, J. A. Tanner, *P Natl Acad Sci USA* **2013**, *110*, 15967-15972.

[81] R. M. Dirkzwager, A. B. Kinghorn, J. S. Richards, J. A. Tanner, *Chemical Communications* **2015**, *51*, 4697-4700.

[82] aL. A. Fraser, A. B. Kinghorn, R. M. Dirkzwager, S. Liang, Y. W. Cheung, B. Lim, S. C. Shiu, M. S. L. Tang, D. Andrew, J. Manitta, J. S. Richards, J. A. Tanner, *Biosensors & bioelectronics* **2018**, *100*, 591-596; bR. M. Dirkzwager, S. Liang, J. A. Tanner, *ACS Sensors* **2016**, *1*, 420-426; cG. Figueroa-Miranda, L. Feng, S. C.-C. Shiu, R. M. Dirkzwager, Y.-W. Cheung, J. A. Tanner, M. J. Schöning, A. Offenhäusser, D. Mayer, *Sensors and Actuators B: Chemical* **2018**, *255*, 235-243; dL. A. Fraser, A. B. Kinghorn, R. M. Dirkzwager, S. Liang, Y.-W. Cheung, B. Lim, S. C.-C. Shiu, M. S. L. Tang, D. Andrew, J. Manitta, J. S. Richards, J. A. Tanner, *Biosensors and Bioelectronics* **2018**, *100*, 591-596.

[83] X. Lin, Q. Chen, W. Liu, J. Zhang, S. Wang, Z. Lin, J.-M. Lin, *Scientific Reports* **2015**, *5*, 9643.

[84] aH. Xu, K. Gorgy, C. Gondran, A. Le Goff, N. Spinelli, C. Lopez, E. Defrancq, S. Cosnier, *Biosensors and Bioelectronics* **2013**, *41*, 90-95; bL.-D. Li, H.-T. Zhao, Z.-B. Chen, X.-J. Mu, L. Guo, *Sensors and Actuators B: Chemical* **2011**, *157*, 189-194.

[85] T. Lim, S. Y. Lee, J. Yang, S. Y. Hwang, Y. Ahn, *BioChip Journal* **2017**, *11*, 109-115.

[86] S. R. Shin, Y. S. Zhang, D.-J. Kim, A. Manbohi, H. Avci, A. Silvestri, J. Aleman, N. Hu, T. Kilic, W. Keung, M. Righi, P. Assawes, H. A. Alhadrami, R. A. Li, M. R. Dokmeci, A. Khademhosseini, *Analytical Chemistry* **2016**, *88*, 10019-10027.

[87] aR. W. Hanson, *Biochemical Education* **1989**, *17*, 86-92; bM. Koupenova, K. Ravid, *Journal of cellular physiology* **2013**, 10.1002/jcp.24352.

[88] aH. Zhang, X. Hu, X. Fu, *Biosensors and Bioelectronics* **2014**, *57*, 22-29; bX. Wei, T. Tian, S. Jia, Z. Zhu, Y. Ma, J. Sun, Z. Lin, C. J. Yang, *Analytical Chemistry* **2016**, *88*, 2345-2352; cY. Zhang, D. Gao, J. Fan, J. Nie, S. Le, W. Zhu, J. Yang, J. Li, *Biosensors and Bioelectronics* **2016**, *78*, 538-546; dH. Fu, J. Yang, L. Guo, J. Nie, Q. Yin, L. Zhang, Y. Zhang, *Biosensors and Bioelectronics* **2017**, *96*, 194-200; eY. Du, C. Chen, M. Zhou, S. Dong, E. Wang, *Analytical Chemistry* **2011**, *83*, 1523-1529.

[89] Q. Wu, H. Shen, H. Shen, Y. Sun, L. Song, *Talanta* **2016**, *150*, 531-538.

[90] J. Yan, M. Yan, L. Ge, J. Yu, S. Ge, J. Huang, *Chemical Communications* **2013**, *49*, 1383-1385.

[91] L. Ge, P. Wang, S. Ge, N. Li, J. Yu, M. Yan, J. Huang, *Analytical Chemistry* **2013**, *85*, 3961-3970.

[92] aL. Wang, G. Musile, B. R. McCord, *Electrophoresis* **2018**, *39*, 470-475; bJ. P. Hilton, T. H. Nguyen, R. Pei, M. Stojanovic, Q. Lin, *Sensors and Actuators A: Physical* **2011**, *166*, 241-246; cJ. Zhou, A. V. Ellis, H. Kobus, N. H. Voelcker, *Analytica Chimica Acta* **2012**, *719*, 76-81; dJ. S. Swensen, Y. Xiao, B. S. Ferguson, A. A. Lubin, R. Y. Lai, A.

J. Heeger, K. W. Plaxco, H. T. Soh, *Journal of the American Chemical Society* **2009**, *131*, 4262-4266.

[93] aY. Ma, Y. Mao, D. Huang, Z. He, J. Yan, T. Tian, Y. Shi, Y. Song, X. Li, Z. Zhu, L. Zhou, C. J. Yang, *Lab on a Chip* **2016**, *16*, 3097-3104; bR. Guider, D. Gandolfi, T. Chalyan, L. Pasquardini, A. Samusenko, C. Pederzolli, G. Pucker, L. Pavesi, *Sensing and Bio-Sensing Research* **2015**, *6*, 99-102.

[94] P. B. Lillehoj, F. Wei, C.-M. Ho, *Lab on a Chip* **2010**, *10*, 2265-2270.

[95] aR. Liu, Y. Huang, Y. Ma, S. Jia, M. Gao, J. Li, H. Zhang, D. Xu, M. Wu, Y. Chen, Z. Zhu, C. Yang, *ACS Applied Materials & Interfaces* **2015**, *7*, 6982-6990; bB. Jin, Y. Yang, R. He, Y. I. Park, A. Lee, D. Bai, F. Li, T. J. Lu, F. Xu, M. Lin, *Sensors and Actuators B: Chemical* **2018**, *276*, 48-56; cB. C. Galarreta, M. Tabatabaei, V. Guieu, E. Peyrin, F. Lagugné-Labarthet, *Analytical and Bioanalytical Chemistry* **2013**, *405*, 1613-1621.

[96] C. Fu, Y. Wang, G. Chen, L. Yang, S. Xu, W. Xu, *Analytical Chemistry* **2015**, *87*, 9555-9558.

[97] K. Keesung, G. Man‐Bock, K. Do‐Hyun, P. Jee‐Won, S. In‐Hong, J. Ho‐Sup, S. Kahp‐Yang, *ELECTROPHORESIS* **2010**, *31*, 3115-3120.

[98] C.-C. Wang, J. W. Hennek, A. Ainla, A. A. Kumar, W.-J. Lan, J. Im, B. S. Smith, M. Zhao, G. M. Whitesides, *Analytical Chemistry* **2016**, *88*, 6326-6333.

[99] I. A. Ges, K. P. M. Currie, F. Baudenbacher, *Biosensors and Bioelectronics* **2012**, *34*, 30-36.

[100] B. J. Sanghavi, J. A. Moore, J. L. Chávez, J. A. Hagen, N. Kelley-Loughnane, C.-F. Chou, N. S. Swami, *Biosensors and Bioelectronics* **2016**, *78*, 244-252.

[101] C. Perreard, F. d'Orlye, S. Griveau, B. Liu, F. Bedioui, A. Varenne, *Electrophoresis* **2017**, *38*, 2456-2461.

[102] aJ. Daprà, L. H. Lauridsen, A. T. Nielsen, N. Rozlosnik, *Biosensors and Bioelectronics* **2013**, *43*, 315-320; bK. Zhang, N. Gan, F. Hu, X. Chen, T. Li, J. Cao, *Microchimica Acta* **2018**, *185*, 181; cD. Desai, M. H. Zaman, *Analytical Methods* **2015**, *7*, 1914-1923.

[103] P. L. Mage, B. S. Ferguson, D. Maliniak, K. L. Ploense, T. E. Kippin, H. T. Soh, *Nature Biomedical Engineering* **2017**, *1*, 0070.

[104] S. Zhan, Y. Wu, L. Wang, X. Zhan, P. Zhou, *Biosensors and Bioelectronics* **2016**, *86*, 353-368.

[105] W. Zhou, R. Saran, J. Liu, *Chemical Reviews* **2017**, *117*, 8272-8325.

[106] J. A. Phillips, Y. Xu, Z. Xia, Z. H. Fan, W. Tan, *Analytical chemistry* **2008**, *81*, 1033-1039.

[107] Q. Shen, L. Xu, L. Zhao, D. Wu, Y. Fan, Y. Zhou, W. H. OuYang, X. Xu, Z. Zhang, M. Song, *Advanced Materials* **2013**, *25*, 2368-2373.

[108] P. Song, D. Ye, X. Zuo, J. Li, J. Wang, H. Liu, M. T. Hwang, J. Chao, S. Su, L. Wang, *Nano letters* **2017**, *17*, 5193-5198.

[109] T. Tian, X. Wei, S. Jia, R. Zhang, J. Li, Z. Zhu, H. Zhang, Y. Ma, Z. Lin, C. J. Yang, *Biosensors and Bioelectronics* **2016**, *77*, 537-542.

[110] aN. C. Seeman, *Journal of Theoretical Biology* **1982**, *99*, 237-247; bN. R. Kallenbach, R.-I. Ma, N. C. Seeman, *Nature* **1983**, *305*, 829.

[111] aP. W. K. Rothemund, *Nature* **2006**, *440*, 297; bB. Wei, M. Dai, P. Yin, *Nature* **2012**, *485*, 623; cL. Qian, Y. Wang, Z. Zhang, J. Zhao, D. Pan, Y. Zhang, Q. Liu, C. Fan, J. Hu, L. He, *Chinese Science Bulletin* **2006**, *51*, 2973-2976.

[112] aE. S. Andersen, M. Dong, M. M. Nielsen, K. Jahn, R. Subramani, W. Mamdouh, M. M. Golas, B. Sander, H. Stark, C. L. P. Oliveira, J. S. Pedersen, V. Birkedal, F. Besenbacher, K. V. Gothelf, J. Kjems, *Nature* **2009**, *459*, 73; bB. Yurke, A. J. Turberfield, A. P. Mills Jr, F. C. Simmel, J. L. Neumann, *Nature* **2000**, *406*, 605; cR.

M. Zadegan, M. D. E. Jepsen, L. L. Hildebrandt, V. Birkedal, J. Kjems, *Small* **2015**, *11*, 1811-1817; dL. Xin, C. Zhou, Z. Yang, D. Liu, *Small* **2013**, *9*, 3088-3091.
[113] S. S. Oh, K. Plakos, Y. Xiao, M. Eisenstein, H. T. Soh, *ACS Nano* **2013**, *7*, 9675-9683.
[114] X. Qu, H. Zhang, H. Chen, A. Aldalbahi, L. Li, Y. Tian, D. A. Weitz, H. Pei, *Analytical Chemistry* **2017**, *89*, 3468-3473.
[115] aJ. Bath, S. J. Green, A. J. Turberfield, **2005**, *44*, 4358-4361; bJ. Mai, I. M. Sokolov, A. Blumen, *Physical Review E* **2001**, *64*, 011102.
[116] T. E. Tomov, R. Tsukanov, Y. Glick, Y. Berger, M. Liber, D. Avrahami, D. Gerber, E. Nir, *ACS Nano* **2017**, *11*, 4002-4008.
[117] O. C. Farokhzad, A. Khademhosseini, S. Jon, A. Hermmann, J. Cheng, C. Chin, A. Kiselyuk, B. Teply, G. Eng, R. Langer, *Analytical Chemistry* **2005**, *77*, 5453-5459.
[118] N. Kolishetti, S. Dhar, P. M. Valencia, L. Q. Lin, R. Karnik, S. J. Lippard, R. Langer, O. C. Farokhzad, *P Natl Acad Sci USA* **2010**, *107*, 17939-17944.
[119] L. Zhang, Y. Wang, C. Ma, P. Wang, M. Yan, *RSC Advances* **2015**, *5*, 24479-24485.
[120] aB. Wu, L.-C. Chen, Y. Huang, Y. Zhang, Y. Kang, D.-H. Kim, *Plasmonics* **2014**, *9*, 801-807; bX. Wei, W. Zhou, S. T. Sanjay, J. Zhang, Q. Jin, F. Xu, D. C. Dominguez, X. Li, *Analytical Chemistry* **2018**, *90*, 9888-9896.