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Lab-on-a-chip systems for hypermethylated DNA detection†

Nishant Mishra (s2252570)

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Hypermethylated DNA is a potential biomarker for different kinds of cancer. It is found in bodily fluids and excreta which make it useful for noninvasive cancer diagnostics. The possibility of such a noninvasive test at the point of care has been investigated in a lab-on-a-chip format

1 Introduction

1.1 Lab-on-a-chip systems

A lab-on-a-chip (LOC) system or a micro total analysis system (μTAS) is a device that integrates fluidic structures, sensing and sometimes electrical circuitry on a single wafer substrate to perform analyses that are traditionally performed in a full scale laboratory. LOC have the advantage of using smaller sample volumes and being able to be used at the point of care. LOCs find their use in environmental, forensic and healthcare applications. Furthermore, LOCs can be used for developing better in vitro models, microreactors or perform experiments on single cells. In this essay the author focuses on LOCs for detection of DNA. An ideal biosensor in a LOC should be specific, stable, independent of environmental factors, fast and linear over the range of concentrations of interest.[1]

1.2 DNA

Deoxyribonucleic Acid (DNA) is one of the fundamental biomolecules that encodes information that is used to make proteins.[2] DNA is made of several nucleotides which in turn consist of a sugar molecule, a phosphate molecule and a nitrogen base.[3] The nucleotides are polymerized into strands by Phosphodiester bonds.[2] Single strand DNAs of DNA can pair or hybridize with complimentary single strands using hydrogen bonds between specific pairs of the four nitrogen bases, adenine (A), cytosine (C), guanine (G), and thymine (T), forming the double helical structure.[4]

2 Hypermethylated DNA

2.1 DNA methylation

DNA methylation is the process by which a methyl (CH_3) group is covalently added to the cytosine base in DNA. DNA methylation is one of the ways that gene expression changes epigenetically rather than due to the order of base pairs.[6] Regions of the DNA with a high number of methylated Cytosine-phosphodiester-Guanine (CpG) sites are not transcribed during the DNA replication process.[7]

CpG islands are genomic regions with a high density of CpG sites, dinucleotide sequences of C at the 5' end followed by G on the 3' end. CpG islands are found in or around 28-72% of promoters[8], regions of the DNA that indicate the start of transcription. In humans and most mammals 60-80% of CpG sites are methylated.[9]

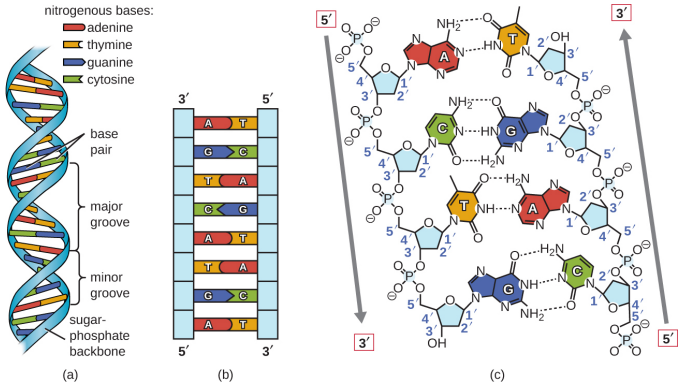

Fig. 1 Structure of DNA[5]

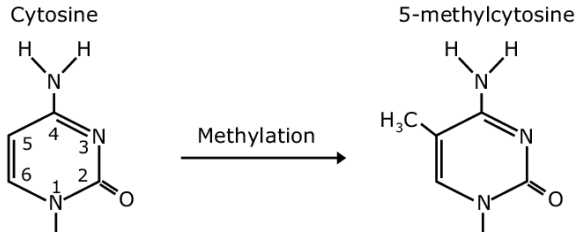

Fig. 2 Methylation of Cytosine[7]

2.1.1 Hypermethylated DNA as a biomarker

DNA methylation is abnormally high in CpG islands. Hypermethylated DNA(hmDNA) has been linked to tumor genesis[10] and has been used as a biomarker for lung[11], prostate[12], colorectal[13] and esophageal[14] cancers. Moreover cell free biomarkers such as hmDNA are of particular interest for use in LOCs as, unlike protein biomarkers, they are specific to different kinds of cancer.

3 DNA enrichment

Higher concentrations of biomarkers are easier to detect as higher signal to noise ratios can be achieved. A few enzymatic reactions to amplify DNA have been described below.

3.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is an enzymatic reaction [15] that allows small sections of DNA to be replicated from small quantities of DNA. [16] Methylated DNA can also be replicated using PCR. [17] Methylation specific PCR allows the amplification of methylated DNA against a background of nonmethylated DNA. [18]

PCR requires the presence of template DNA- the DNA to be replicated, primers- nucleic acids required to initiate the replication, nucleotides (A T G and C)- the building blocks of DNA, and DNA polymerase- an enzyme that synthesizes DNA from nucleotides. [19]

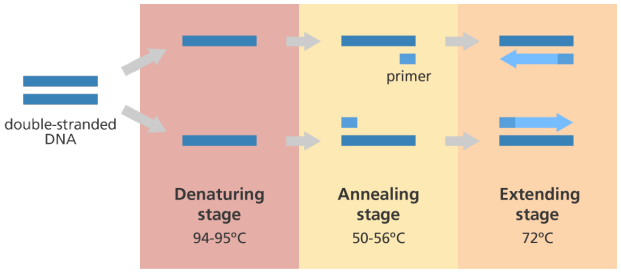

Fig. 3 Main steps of PCR [19]

PCR consists of three different steps at different temperatures: denaturing(15-30s at 94-95°)- heating double stranded DNA to separate it into single strands, annealing(10-15s at 50-65°)- attaching primers to the template at a lower temperature, and extending(1 minute per 1000 base pairs being copied at 37°)- formation of a new DNA strand using DNA polymerase. These three steps are repeated 20-40 times to obtain copies of the DNA of interest, with the amount of DNA doubling with every cycle. An improvement on the traditional PCR technique is the Real-Time PCR (RT-PCR) [20] which is faster, has higher sensitivity and selectivity and is more standard.

In the past two decades many groups developed PDMS [21 22], SU-8 [23] and glass [24] based microreactors enabling PCR on a LOC platform. These groups combine microfluidics along with electrical joule heating and temprature sensing.

Although DNA amplifications and good temperature control has been achieved, challenges include the lack of long duration pumps for PDMS and the relative long duration(approx 40 mins) of the reaction for a point of care test and bubble generation in the microfluidic chips.

3.2 Loop-mediated Isothermal Amplification

Loop-mediated isothermal amplification(LAMP) is a DNA amplification technique that takes place at a constant temperature. The lack of thermal cycling makes LAMP a continuous process that is faster and cheaper than traditional PCR amplification. [25]

LAMP consists of a non cyclic template DNA elongation, and cyclic step where new primers bind and elongate the loop region. [27]

LAMP on a chip is advantageous over PCR on a chip as the isothermal reaction is easier to control and thus avoids bubble generation [28]. LAMP on a LOC platform has been used for detection of viral [28 29] and bacterial [30 31] DNA. LAMP can also be used

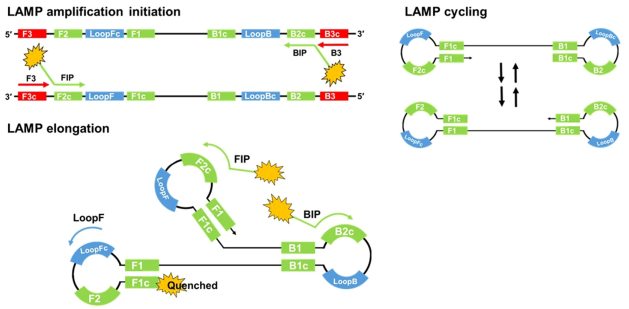

Fig. 4 Main steps of LAMP [26]

to amplify methylated DNA. [32 33 34]

Although LAMP offers several advantages over PCR, primer design limits the segments of DNA that can be amplified. [25] So far LAMP based LOCs for hmDNA have not been reported in the literature.

3.3 Rolling Circle Amplification

Another isothermal DNA replication recation is the Rolling Circle Amplification(RCA) in which nucleotides are added in a continuous cycle. [35]

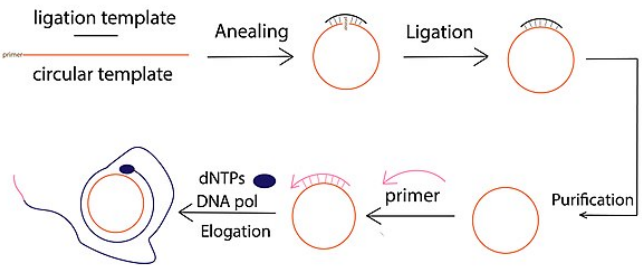

Fig. 5 Main steps of RCA [26]

Though this technique is based on replication of circular plasmid DNA, recently it has been demonstrated to be used for replication of nucleosomal DNA. [36] Since this technique is relatively new, it has not yet been integrated into a LOC.

4 Extraction and separation of DNA

hmDNA is a cell free nucleic acid biomarker and is found in various bodily fluids and excreta such as blood, saliva, urine and feces. This allows for possibilities of liquid biopsies as opposed to traditional surgical biopsies. LOCs have been developed to detect hmDNA in saliva [37]. Extraction of enrichment of hmDNA has also been performed on a LOC format.

De et al. Fabricated a LOC to capture enrich and purify sub ng per mL concentrations of hmDNA. [38] 95% of the DNA was captured and enriched to 28X its original concentration on chip. They describe a 2D sillicon micropillar array coated with methyl binding domain proteins.

On the other hand Shin et al. propose a dimethyl adipimidate

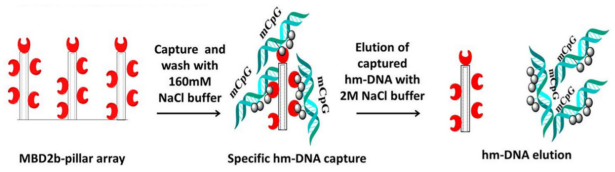

Fig. 6 Schematic of DNA capture and elutation by Methyl binding protein [38]

(DMA) based extraction and elutation process that overcomes the complexity of manufacturing of structures such as nanopillar arrays. [39] The DMA network provides a high surface area structure for the capture of hmDNA, in addition to this, the capture is reversable at higher pH.

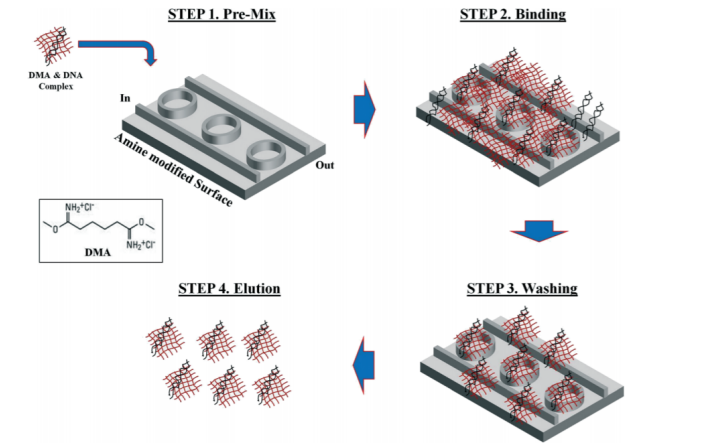

Fig. 7 Schematic of DNA capture and elutation by dimethyl adipimidate [39]

5 DNA sensing

After amplification and extraction, the DNA needs to be detected and quantified. DNA sensors consist of a biorecognition element (most often a complimentary strand of ssDNA) and a transducer that converts the chemical recognition of the complimentary DNA into the physical domain. [40]

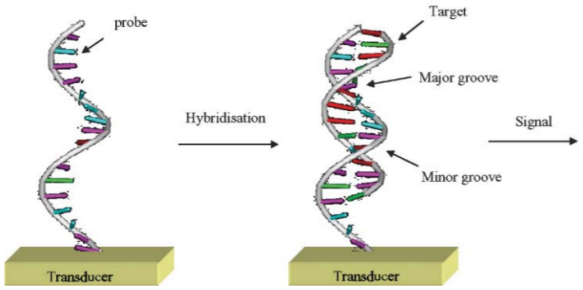

Fig. 8 Specific capture and transduction of signal into physical domain [40]

Optical methods such as florescence and Surface Plasmon resonance are briefly discussed but the main focus of this paper lies in electrochemical sensor elements. Optical methods such as Surface Plasmon Resonance [41] cause a change in the refractive index for a label free assay. Fluorescence is a technique that causes radiation after absorption of higher energy of light. [42]

5.1 Electrochemical Methods

Although optical have their own merits and application, electrochemical sensors are prefered in LOC format as they are directly able to convert the biochemical signal into an electrical signal that can be processed easily.

5.1.1 Potentiometric Sensors

When a conductor is placed in an electrolyte solution, there is a tendency for electrons to flow to lower energy states (into or out of the working electrode). This causes a resultant reduction or oxidation current. At equilibrium, the working electrode is at a potential called the nernst potential where no current flows. Measuring the nernst potential gives us information about analyate concentration in solution. This method is unfortunately not selective to specific ions but selection can be made using an ion selective electrode. [43] Difficulties in miniaturization of reference electrodes is also one of the main drawbacks of this method. Nonetheless, a modification of this principal, called cyclic voltametry has been used to detect femtomolar concentrations of hmDNA. [44] Here the potential is deviated from the nernst potential and current characteristics are observed. The sensor system uses composites with a streptavidin-biotin chemistry and anti-5-methylcytosine antibodys to capture hmDNA.

5.1.2 Amperometric sensors

Amperometric sensors apply a potential across a working and a reference electrode and measure the current through the solution. This current is proportional to the ionic current due to mass transport of electrochemical species to the electrodes. [45] Many biosensors including the well known glucose sensor make use of amperometric sensors for transduction of the signal. Enzymatic glucose sensors use glucose oxidase to indirectly measure the concentration of glucose by measuring the concentration of H_2O_2 amperometrically. [46] In 2018 an amperometric sensor for hmDNA was described by Povedano et al. uses surface modified magnetic beads to capture and concentrate hmDNA [47].

It uses a similar mechanism of enzymatic reduction of hydrogen peroxide due to hydroquinone. Amperometric measurement of hydrogen peroxide concentration gives a measurement of hmDNA concentration. This method was able to be used for picomolar concentrations of hmDNA.

Unfortunately since amperometric sensors depend on mass transport, their applications are limited in LOCs measuring flowing samples. Amperometric current is also dependent on the area of the electrodes which can cause decreased signal to noise ratio as miniaturization increases.

5.1.3 Electrolyte conductivity or impedance based sensors

The final major class of electrochemical sensors is the electrolyte conductivity sensor, which measures the non redox conductivity of the solution to an alternating excitation. Electrochemical Impedance Spectroscopy (EIS) has been used to detect hmDNA by Povedano et al. in the same publication. [47]

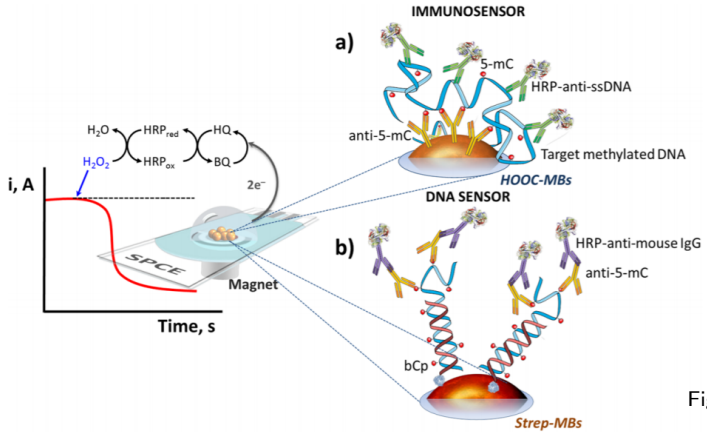

Fig. 9 Current is porportional to concentration of H_2O_2. H_2O_2 reduction is mediated by Horseradish Peroxidase(HRP) which in turn is dependent on the Hydroquinone/Benzoquinone (HQ/BQ) redox reaction[47]

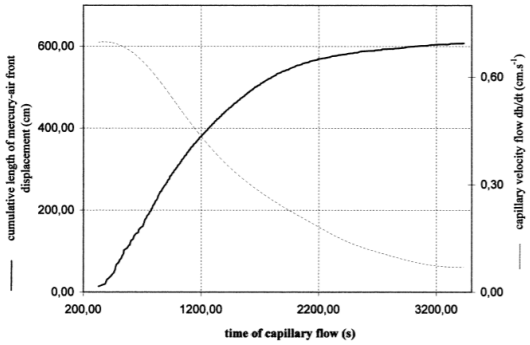

Fig. 10 Time dependence on capillary flow rates[53]

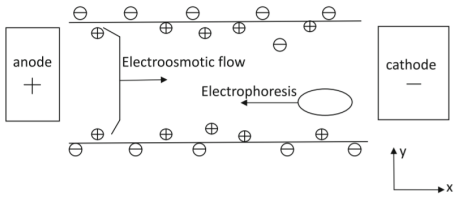

Fig. 11 Electrokinetic forces on ions in the double layer and on a negatively charged DNA molecule[55]

6 Microfluidics: From biosensors to Lab-on-a-Chip systems

Integration of flow of reagents and analytes with the biosensor forms a LOC system. Practical considerations for LOC design including the channel materials, the pumping systems and mass transport and reaction kinetics have been examined here.

6.1 Channel Materials

Microfluidic channels are usually made of polymers[48], glass or silicon[49]. Each of these materials has its advantages. Silicon is the most expensive but traditional MEMS fabrication techniques can be used to form microchannels. The transparency of glass can be advantageous in optical biosensor based LOCs. Polymer based materials are cheap and easy to fabricate. The surface properties of the materials such as surface energy (hydrophilicity and hydrophobicity) can have an impact on the flow[50] and biofouling.[51]

6.2 Pumping systems

Microfluidic pumping systems are required to move the solution to be analyzed to the biosensor element. Pressure driven flow which uses syringe pumps to drive the flow. Though the setup is simple this method does not scale well with miniaturization as the hydraulic resistance of the system increases.[52] Another popular method is using capillary forces where the fluid moves through the channel due to the adhesive forces in channel wall and fluid being stronger than the cohesive forces between the fluid molecules.[54] Capilary flow has the disadvantage that flow rates are proportional to the square root of time which might be undesirable for a fast diagnostics platform.[53] Finally electroosmotic flow (EOF), which uses electrical fields to drive ions in the diffuse double layer of the liquid-channel interface can be used.[55] EOF velocities and directions can be tuned by applying a second field.[56] EOF scales well with smaller dimensions.

6.3 Diffusion, convection, and surface reactions

Flow rates in microchannels can be optimized to enable maximum surface capture of target biomarkers. The ideal flow rates depend on the rate of mass transport- diffusion or convection, as well as the binding reaction kinetics. Some important dimensionless constants that charecterize the system are the Peclet and Dahmkohler number.[57] The Peclet number determines weather

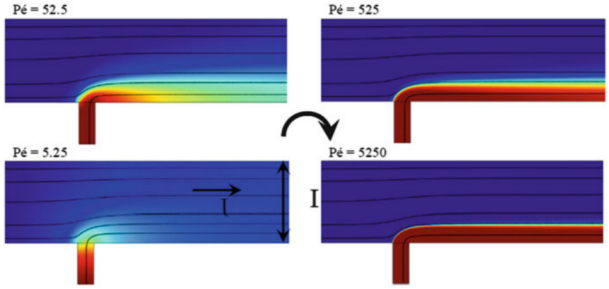

Fig. 12 Influence of increasing peclet number[58]

the depltion zone grows or propagates upstream from the sensor surface at a given flow rate[58]. The Dahmkohler number on the other hand characterizes the ratio of the biomarker that has absorbed on the surface versus the amount of biomarker that diffuses to the surface.

7 Conclusion

The origin of hypermethylated DNA, its role in gene expression and its potential as a cancer biomarker are briefly discussed.

Different strategies for DNA enrichment were discussed. The drawbacks of PCR can be addressed by newer gene amplification

techniques such as LAMP and RCA that operate at a constant temprature.

Solid phase extraction methods using nanopillar arrays was discussed, an alternate polymer network based method was also elucidated to overcome the complexity of fabrication of such nanostructures.

The importance of selecting the right channel materials and dimensionless constants like dahmkohler and peclet numbers that characterize the microfluidic system are discussed in brief. Generally a combination of pumping systems are used to overcome the individual disadvantages.

Working, pros and cons of each of the electrochemcial sensing methods have been discussed. Amperometric detection seems to be the right choice for cheap and miniaturized systems.

Some of the literature around all these aspects of a lab on a chip system have been reviewed to facilitate design an LOC for hmDNA detetction.

Notes and references

1 B. Rafique, M. Iqbal, T. Mehmood and M. A. Shaheen, *Sensor Review*, 2019, **39**, 34–50.

2 R. Lewis, *Encyclopedia of Genetics*, Elsevier, 2001, pp. 1171–1180.

3 D. A. Vallero, *Environmental Biotechnology*, Elsevier, 2010, pp. 99–165.

4 J. D. Watson and F. H. Crick, *Nature*, 1953, **171**, 737–738.

5 N. Parker, M. Schneegurt, A. T. Tu and B. M. Forster, *Microbiology (Open textbook library.)*, OpenStax College, Rice University, 2016.

6 T. Chen, *Progress in Molecular Biology and Translational Science*, 2011, **101**, 335–348.

7 P. Kumar and U. Mina, *Life Sciences, Fundamentals and Practice, Part II*, 2016.

8 *Proceedings of the National Academy of Sciences of the United States of America*, 2006, **103**, 1412–1417.

9 K. Jabbari and G. Bernardi, *Gene*, 2004, **333**, 143–149.

10 S. B. Baylin and J. G. Herman, *Trends in Genetics*, 2000, **16**, 168–174.

11 S. A. Belinsky, *Nature Reviews Cancer*, 2004, **4**, 707–717.

12 P. J. Bastian, S. Yegnasubramanian, G. S. Palapattu, C. G. Rogers, X. Lin, A. M. De Marzo and W. G. Nelson, *European Urology*, 2004, **46**, 698–708.

13 D. R. Wang and D. Tang, *World Journal of Gastroenterology*, 2008, **14**, 524–531.

14 Z. Jin, A. Olaru, J. Yang, F. Sato, Y. Cheng, T. Kan, Y. Mori, C. Mantzur, B. Paun, J. P. Hamilton, T. Ito, S. Wang, S. David, R. Agarwal, D. G. Beer, J. M. Abraham and S. J. Meltzer, *Clinical Cancer Research*, 2007, **13**, 6293–6300.

15 K. Mullis, F. Faloona, S. Scharf, R. Saiki, G. Horn and H. Erlich, *Cold Spring Harbor Symposia on Quantitative Biology*, 1986, **51**, 263–273.

16 L. Garibyan and N. Avashia, *Journal of Investigative Dermatology*, 2013, **133**, 1–4.

17 Z. J. Liu and M. Maekawa, *Analytical Biochemistry*, 2003, **317**, 259–265.

18 J. L. Ku, Y. K. Jeon and J. G. Park, *Methods in Molecular Biology*, 2011, **791**, 23–32.

19 *What is PCR (polymerase chain reaction)?*, 2016, `https://www.yourgenome.org/facts/what-is-pcr-polymerase-chain-reaction`.

20 I. M. Mackay, *Nucleic Acids Research*, 2002, **30**, 1292–1305.

21 Q. Xiang, B. Xu and D. Li, *Biomedical Microdevices*, 2007, **9**, 443–449.

22 N. Ramalingam, H. B. Liu, C. C. Dai, Y. Jiang, H. Wang, Q. Wang, K. M Hui and H. Q. Gong, *Biomedical Microdevices*, 2009, **11**, 1007–1020.

23 J. El-Ali, I. R. Perch-Nielsen, C. R. Poulsen, D. D. Bang, P. Telleman and A. Wolff, *Sensors and Actuators, A: Physical*, 2004, **110**, 3–10.

24 S. K. Jha, R. Chand, D. Han, Y. C. Jang, G. S. Ra, J. S. Kim, B. H. Nahm and Y. S. Kim, *Lab on a Chip*, 2012, **12**, 4455–4464.

25 M. M. Parida, S. Sannarangaiah, P. K. Dash, P. V. Rao and K. Morita, *Reviews in Medical Virology*, 2008, **18**, 407–421.

26 P. Hardinge and J. A. Murray, *Scientific Reports*, 2019, **9**, 1–13.

27 T. Notomi, Y. Mori, N. Tomita and H. Kanda, *Journal of Microbiology*, 2015, **53**, 1–5.

28 X. Fang, Y. Liu, J. Kong and X. Jiang, *Analytical Chemistry*, 2010, **82**, 3002–3006.

29 X. Fang, H. Chen, S. Yu, X. Jiang and J. Kong, *Analytical Chemistry*, 2011, **83**, 690–695.

30 J. Luo, X. Fang, D. Ye, H. Li, H. Chen, S. Zhang and J. Kong, *Biosensors and Bioelectronics*, 2014, **60**, 84–91.

31 D. M. Tourlousse, F. Ahmad, R. D. Stedtfeld, G. Seyrig, J. M. Tiedje and S. A. Hashsham, *Biomedical Microdevices*, 2012, **14**, 769–778.

32 V. J. Bailey, H. Easwaran, Y. Zhang, E. Griffiths, S. A. Belinsky, J. G. Herman, S. B. Baylin, H. E. Carraway and T. H. Wang, *Genome Research*, 2009, **19**, 1455–1461.

33 E. Olkhov-Mitsel, D. Zdravic, K. Kron, T. Van Der Kwast, N. Fleshner and B. Bapat, *Scientific Reports*, 2014, **4**, 1–8.

34 F. Zerilli, C. Bonanno, E. Shehi, G. Amicarelli, D. Adlerstein and G. M. Makrigiorgos, *Clinical Chemistry*, 2010, **56**, 1287–1296.

35 M. M. Ali, F. Li, Z. Zhang, K. Zhang, D. K. Kang, J. A. Ankrum, X. C. Le and W. Zhao, *Chemical Society Reviews*, 2014, **43**, 3324–3341.

36 C. L. van Emmerik, I. Gachulincova, V. R. Lobbia, M. A. Daniëls, H. A. Heus, A. Soufi, F. H. Nelissen and H. van Ingen, *Analytical Biochemistry*, 2020, **588**, year.

37 B. L. Ziober, M. G. Mauk, E. M. Falls, Z. Chen, A. F. Ziober and H. H. Bau, *Head and Neck*, 2008, **30**, 111–121.

38 A. De, W. Sparreboom, A. van den Berg and E. T. Carlen, *Biomicrofluidics*, 2014, **8**, year.

39 Y. Shin, A. P. Perera, C. C. Wong and M. K. Park, *Lab on a Chip*, 2014, **14**, 359–368.

40 K. J. Odenthal and J. J. Gooding, *Analyst*, 2007, **132**, 603–610.

41 M. Nazmul Islam, S. Yadav, M. Hakimul Haque, A. Munaz, F. Islam, M. S. Al Hossain, V. Gopalan, A. K. Lam, N. T. Nguyen and M. J. Shiddiky, *Biosensors and Bioelectronics*, 2017, **92**, 668–678.

42 M. Dadmehr, M. Hosseini, S. Hosseinkhani, M. R. Ganjali, M. Khoobi, H. Behzadi, M. Hamedani and R. Sheikhnejad, *Biosensors and Bioelectronics*, 2014, **60**, 35–44.

43 R. De Marco, G. Clarke and B. Pejcic, *Electroanalysis*, 2007, **19**, 1987–2001.

44 M. Daneshpour, L. S. Moradi, P. Izadi and K. Omidfar, *Biosensors and Bioelectronics*, 2016, **77**, 1095–1103.

45 J. Wang, *Journal of Pharmaceutical and Biomedical Analysis*, 1999, **19**, 47–53.

46 S. J. Updike and G. P. Hicks, *Nature*, 1967, **214**, 986–988.

47 E. Povedano, E. Vargas, V. R. V. Montiel, R. M. Torrente-Rodríguez, M. Pedrero, R. Barderas, P. S. Segundo-Acosta, A. Peláez-García, M. Mendiola, D. Hardisson, S. Campuzano and J. M. Pingarrón, *Scientific Reports*, 2018, **8**, year.

48 H. Becker and C. Gärtner, *Analytical and Bioanalytical Chemistry*, 2008, **390**, 89–111.

49 C. Iliescu, H. Taylor, M. Avram, J. Miao and S. Franssila, *Biomicrofluidics*, 2012, **6**, year.

50 M. Kalin and M. Polajnar, *Tribology Letters*, 2013, **52**, 185–194.

51 Q. Zhao, Y. Liu, C. Wang, S. Wang and H. Müller-Steinhagen, *Chemical Engineering Science*, 2005, **60**, 4858–4865.

52 K. W. Oh, K. Lee, B. Ahn and E. P. Furlani, *Design of pressure-driven microfluidic networks using electric circuit analogy*, 2012.

53 A. Cerepi, L. Humbert and R. Burlot, *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 2002, **206**, 425–444.

54 H. Noh and S. T. Phillips, *Analytical Chemistry*, 2010, **82**, 4181–4187.

55 K. Yan, Y. Z. Chen, J. Han, G. R. Liu, J. S. Wang and N. G. Hadjiconstantinou, *Microfluidics and Nanofluidics*, 2012, **12**, 157–163.

56 E. J. Van Der Wouden, T. Heuser, D. C. Hermes, R. E. Oosterbroek, J. G. Gardeniers and A. Van Den Berg, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2005, pp. 110–116.

57 T. M. Squires, R. J. Messinger and S. R. Manalis, *Nature Biotechnology*, 2008, **26**, 417–426.

58 J. Castillo-León and W. E. E. Svendsen, *Lab-on-a-Chip Devices and Micro-Total Analysis Systems: A Practical Guide*, 2015, p. 1.