Proceedings of the ASME 2010 3rd Joint US-European Fluids Engineering Summer Meeting and 8th International Conference on Nanochannels, Microchannels, and Minichannels
FEDSM-ICNMM2010
August 1-5, 2010, Montreal, Canada

FEDSM-ICNMM2010-30174

ENTERING A NEW ERA OF DIAGNOSIS

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ABSTRACT

Looking at the development of diagnostics from prehistorical days up to know and even further visioning into the future the shamans of the old days were slowly replaced by the early "all-round" doctor having first simple diagnostical and surgery possibilities, changing to nowadays specialized physicians doing the diagnoses based on analytical results provided by decentralized specialized labs. Future visions present doctors offices harboring small instruments that allow the physicians to do analyses directly as fast and as minimally or even non-invasive as possible advantageously combined with a connection to a smart health care database providing anamnesis and providing possible therapeutical measures.

Already in the 1960s' science fiction series Star Trek the spaceship crew used very small instruments for fast, non-invasive diagnosis and treatment. Although, such analyzers are future vision actual developments lead to less and less complex and small systems. Using micro- and nano-technologies manifold approaches addressing so-called "Lab-on-a-chip (LoC)" or "micro total analysis systems (μTAS)" where described during the last two decades. Huge progress can be seen in miniaturization not only of electronics but also of mechanics. While presently, table-top systems reach the market handheld systems providing complete analysis from sample taking to result are rare.

Presently, often complex sample preparation methods have to be performed to reach the sensitivity and robustness needed for reliable results. In addition, specific disease markers are still missing that give clear conclusions about health status. In this field, intensive research is going on identifying new better and more specific markers for fast and easy reliable determination of diseases, infections, predispositions and more. Having markers available where each marker gives a non-misleading conclusion that a person will have or already has a certain disease, being able to determine these markers directly from the sample without complex sample preparation steps and having instruments available being preferably portable and applicable by non-specialists such a vision is getting closer.

The actually developed miniaturized instruments are an important step towards the envisioned future systems demonstrating the basic proof of concept and thereby heralding a new era of diagnosis.

INTRODUCTION

In prehistorical times our ancestors used leafs, roots, crust, insects, animal parts, dung, bones or even mineral stones prepared as drink, mash, wet pack, or just as diagnosis tool often in combination with ceremonial dances to fight against the "bad evils" that cause suffering. Some of these practices can still be found today. In the middle age when arteriotomy was the predominant therapy for most diseases people slowly got aware of the fact that diseases correlate somehow with the environment and behavior in terms of cleanliness. For example, bacteria were first observed in the 17th century[1] but it took even until the 19th century to come to the conclusion that these organisms cause many diseases[2].

In the earliest days of modern medical practice the physician did all diagnostic tests, surgeries and therapies himself at his office. With the emergence of modern diagnostics using biochemical, micro- and molecular biological technologies specific, selective and sensitive but often complex and laborious analysis methods were established and more and more analysis were done decentralized in big central labs. Time from sample taking to the result reported to the patient therefore usually takes up to several weeks. Until this moment the patient has to count on the physicians experience and his choice of medication. Often, physicians give drugs that are not necessarily indicated, e.g. broad-spectrum antibiotics, just to be sure to hit the cause of disease. In other cases, patients have to wait for the results until they get treated at all. Both situations are not favorable since in the first case more and more resistant germs lead to more and more ineffective treatments while in the

second case the patient still suffers and, in addition, may get into physiological stress.

In both cases it is advantageous to get the result during the patients stay in the doctor's office. Having small automated systems the analyses can be done on-demand and the adequate medical treatment can immediately be started. Hereby, it is important that these systems show at least the same performance in terms of sensitivity, reliability and reproducibility as the standard methods used in central labs. Nevertheless, it has to be mentioned that it is not necessary to have all results immediately.

MINIATURISATION

For several applications fast, easy-to-handle analysis kits are already on the market. Most prominent examples are glucose test strips for diabetes patients or pregnancy tests. These are just two of many examples where lateral flow test strips are used. All of these tests have in common that the analysis process steps are very simple. Unfortunately, most analyses need more complex process steps that cannot be realized within a just capillary-driven fleece. Therefore, actively driven systems have to be realized. A major challenge is the transfer of standard assays into automated and miniaturized systems wherefore the assays have to be simplified as much as possible.

Applying microtechnology and microfluidics in particular has the potential to make a major contribution to decentralising and simplifying medical/diagnostic testing. Lab-on-a-chip systems that integrate several lab functions within a single polymer substrate open up new perspectives with respect to early diagnosis, disease therapy and monitoring of infectious, autoimmune and cancer diseases. Automated analyses of complex fluids with high efficiency and speed used by non-skilled operators lead to time and labour saving tools for primary use within medical practice, clinical oncology and speciality laboratories.

Transferring analysis of biological samples into microfluidic systems is not trivial, due to the complexity of the samples. Required sensitivity and specificity as well as time to result and envisaged costs significantly determine the choice of technologies to be applied. Two major carriers of information are present in biological samples – nucleic acids and proteins. In order to get access to the information needed the carriers have to be separated, isolated, concentrated, purified, amplified, modified and finally detected and analysed. Sample preparation thereby plays a major role but not astonishingly, most current micro total analysis systems (μTAS) still start analysis from a pre-treated sample. The multitude of required functionalities, the resulting complexity of integration and a standardisation level that still needs to be improved clearly indicate that the development of highly integrated LoC systems still requires considerable R&D efforts until maturity is achieved.

Besides cardiovascular diseases and cancer which are at present the highest causes of death in the world infectious diseases, like aftosa or the bird or swine flue, show up again and again and demonstrate the need for new fast and efficient point-of-care analysis tools. Therefore, the development of cheap and fast technologies for diagnosis and therapy monitoring is one of the most challenging topics in modern medical science.

GERMS IN AQUEOUS SOLUTION

Determination of bacteria in aqueous solutions like urine, water or samples from air samplers is a common application where a volume of several milliliters has to be examined for a possible bacterial load. Conventionally, the sample is centrifuged for separation and concentration of bacteria followed by spin-tube based isolation of the nucleic acids for subsequent amplification and detection.

Fig. 1 Microfluidic chip-based system for isolation and lysis of bacteria followed by nucleic acid purification. The disposable chip (top) shows filters for cell accumulation and DNA binding, Kenics mixers, buffer reservoir channels and turning valves for connecting channels. The operational device (bottom) contains electronics for process control on chip including heaters. Fluid transport is realized by a syringe pump.

Fig. 1 shows an injection molded chip made of COP (cyclo-olefin polymer) and the corresponding instrument for the isolation and lysis of bacteria followed by nucleic acid purification. Up to 10 ml of an aqueous solution is pushed into the system via a syringe. Bacteria are retained by a filter while

fluid is transported to the waste reservoir. Bacteria get resolved in detergent-containing lysis buffer and lysis is supported by heating. Lysed sample is then mixed with a high salt buffer to adjust conditions for nucleic acid binding to a silica matrix. After incubation of the silica matrix with the sample the matrix gets washed with two washing buffers, followed by a drying step to remove excess ethanol. Nucleic acids are eluted with water and dosed into micro titer plate wells for subsequent PCR analysis.

To mix the buffers (several 100 µl) efficiently so-called Kenics mixers are integrated into the channels. Buffers needed have to be filled into the chip prior to the analysis. Therefore, metering channels are integrated for holding the appropriate volumes. Fluid control is realized by several turning valves and light barriers for fluid positioning. Filters are fixed by thermal welding in allotted cavities and silica matrices are clamped.

Reagents used are taken from the PrestoPlant D Kit from Molzym[3], silica matrices are provided separately by Molzym. Experiments show that using the developed chip system 10^3 E. coli can be isolated from 10 ml water and determined via realtime PCR (Fig. 2).

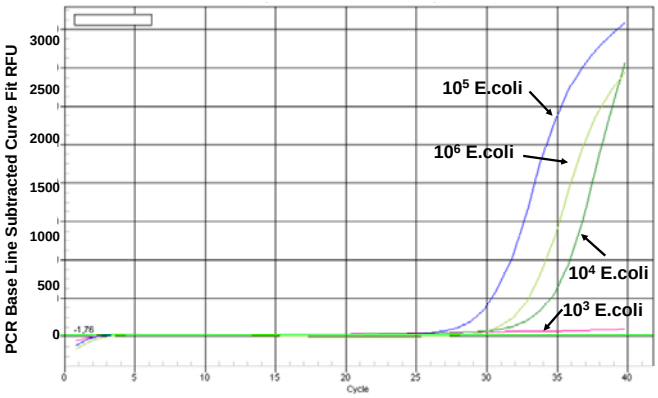

Fig. 2 Realtime PCR analysis of isolated DNA. DNA purified from aqueous solutions using the system shown in Fig. 1 was analyzed in realtime PCR.

HPV IN CERVICAL SMEARS

Second highest cause of death worldwide is cancer (WHO Fact sheets). With about 500.000 new patients per year cervix cancer is one of the major cancer worldwide. Since human papilloma viruses (HPV) are the activators of this disease[4] their determination as part of a thorough check-up for cancer is advantageous. At present cervix smears get analyzed in central labs taking several weeks. An analysis directly at the doctor's office is preferable, to start immediately with the therapy advised. Determination of HPV via mRNA instead of viral DNA as done usually today reduces false positive results since only active viruses are analysed. Already determined in an early stage via expression of specific tumour genes persistent HPV infections can be treated appropriately.

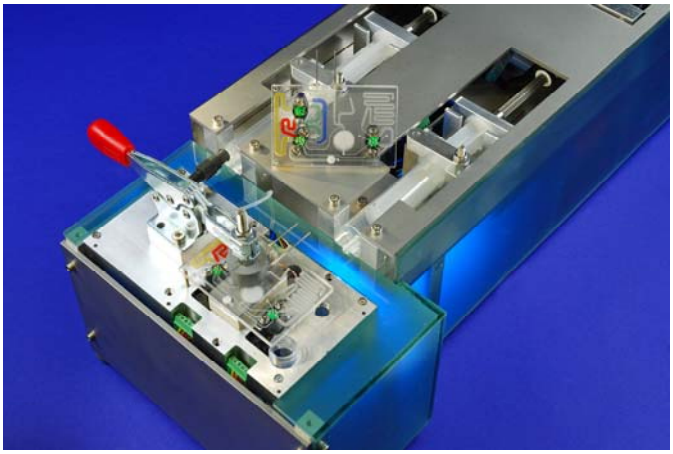

Fig. 3 Automated chip system for the isolation of viral mRNA from cervical smears. The chip comprises two filter membranes (1. cell collection, 2. RNA binding) and several turning valves connecting buffer storage reservoirs with processing channels. The operating device harbors the sample syringe, an additional syringe for fluid transport within the chip and the electronics.

Using the fully automated chip-based system shown in Fig. 3 mRNA from cervical smear samples can be isolated for subsequent analysis (see Tabel 1). Extracted cell solution from cervical smear swabs gets introduced into the system by a syringe. Cells are collected on a filter and lysed with Guanidinium Isothiocyanate. The lysate is guided to a silica membrane where total RNA/DNA is bound, washed and isolated. The purified nucleic acids are then transferred to an amplification chip where specific amplification and analysis (Nucleic Acid Sequence-Based Amplification; NASBA) of several mRNA-sequences of potentially dangerous cancer-relevant HPV subtypes is performed[5]. Both modules are envisaged to be combined in a single LoC-system in near future. With this combined system gene expression profiling can be performed at the doctor's office thereby allowing fast diagnosis and immediate therapy.

Cell line /# of Cells	CaSki HPV16	MS751 HPV45	HeLa HPV18
50.000	positive	positive	positive
5.000	positive	positive	positive
500	positive	positive	positive
50	positive	positive	positive
5	positive	negative	positive

Table 1 Analysis of purified HPV-mRNA from 5-50.000 cells from different human cell lines with NASBA.

By changing this system moderately, e.g. different filter materials, different binding matrices as well as different reagents, also other complex biological samples can be processed. In addition, other amplification and detection methods can be combined with the sample preparation module.

Other possible applications can be found in food and feed analyses, personalized medicine as well as in other Point-of-care diagnostic and forensic applications.

TUMOR CELLS CIRCULATING IN BLOOD

Circulating tumor cells (CTC) are cells that invade the bloodstream and cause distant metastases. The existence of CTCs in blood before primary diagnosis, the evidence of significant levels of CTCs in blood in case of relapse as well as the persistence of CTCs in patients even after primary tumour dissection lead to intensive studies on their potential relevance for early diagnostis and prognosis[7].

The detection of occult metastases, e.g. detection of CTCs, could have a substantial clinical impact on the optimal disease management of cancer patients since undetected micro-metastases can contribute to the failure of primary treatment. Improved methods for direct determination of the presence of and changes in the number of residual malignant cells after surgery may be useful for monitoring of the therapy efficiency. The crucial advantage of a blood test compared to invasive methods is in the safety and potential frequency of examinations. The detection of CTCs is very challenging since their levels in peripheral blood are very low, e.g. down to one cell per ml. Accordingly, an enrichment of these cells is necessary. Numerous methods for cell enrichment based on either immunomagnetic cell separation, e.g. Dynabeads (Invitrogen), or flow cytometry exist.

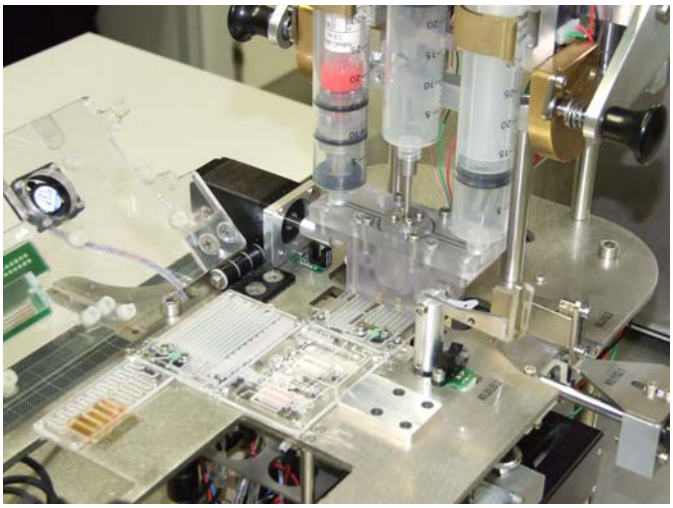

Fig. 4 Microsystem for automated isolation of circulating tumor cells from whole blood samples based on disposable polymer chips. The chip system comprises an incubation module, an amplification module, a buffer storage module and a detection module. The operating device shows all mechanical and electronic components needed for process control.

Fig. 4 shows a microfluidic chip-based platform for the isolation of rare cells (e.g. CTCs) from 7.5 ml peripheral blood and characterisation via gene expression profiling. It integrates modules for immunomagnetic cell enrichment, mRNA isolation followed by multiplex RT and cDNA amplification, and parallel electrochemical detection of the amplicons to determine gene expression profiles. A panel of about 21 mRNA markers has been chosen to allow thorough characterisation of the isolated CTCs.

The sample analysis starts with the incubation (cell isolation) module that performs mixing, bead attraction, exchange of buffers and, finally, transport of cell/bead-complexes and free beads to the subsequent microsystem. The macro-to-micro interface is realised with a simple slide valve. Once transferred to the microsystem the cell/bead-complexes and free beads are retained in a small reservoir on the separation chip with a magnet underneath. Following isolation the CTCs are lysed, their mRNA is converted to cDNA and pre-amplified and, finally, the DNA is amplified via Multiplex Ligation dependent Probe Amplification (MLPA). The complex standard MLPA method has been simplified and miniaturised to allow automated performance on chip. In the final detection module, the gene expression profiles of the isolated cells are determined by analysing the PCR products on an array of electrochemical genosensors. Isolation and analysis of down to five spiked MFC7 cells was shown.

By adapting this system, e.g. changing the sample, the antibodies and the primers, isolation of other (rare) targets can be realised, e.g. fetal cells from maternal blood, disseminated tumour cells (DTCs) in bone marrow, stem cell isolation etc.

INFECTIOUS RESPIRATORY TRACT DISEASES

Respiratory tract infections are still the leading cause among infectious diseases and therefore have a considerable impact on the health budget[6]. Although huge progress is achieved in identifying pathogens especially by applying modern molecular biological methods the number of new infections does not sustainably decrease. In contrast, due to the growing number of antibiotic-resistant strains it is suspected that the numbers will grow in coming years. Here again, early diagnosis followed by personalised efficient therapy of infectious diseases (e.g. respiratory diseases, meningitis, sepsis) can lead to considerable reduction of costs in health care.

Fig. 5 shows an integrated Lab-on-a-chip system which allows performing all diagnostic process steps for pathogen analysis of respiratory viruses from nasopharyngeal samples. It is composed of three parts: a disposable microfluidic processing cartridge made from COP (cycloolefin polymer), a reagent cartridge bearing liquid buffers for 24 analyses and an operating device that controls all processes done within the processing cartridge (fluid movement, metering and mixing, and heat management). On the disposable chip several reagents are stored freeze-dried as are proteinaseK, magnetic beads for RNA/DNA isolation, PCR ingredients and the LiquiChip beads and streptavidin-R-phycoerythrin for labelling. The core of the instrument is a construction based on rotating heating bars allowing for fast cycling times. With a volume of 120 µl this set-up allows a PCR with 30 cycles within one hour.

To start the analysis the user introduces the sample into the lysis chamber of the microfluidic chip, inserts the chip into the device and closes this. Within the device the sample gets mixed with buffers for lysis. Solid phase extraction for RNA purification is performed using magnetic beads. Subsequent reverse transcription is followed by QIAplex PCR technology and, finally, labelling of the nucleic acids by hybridisation with LiquiChip beads and streptavidin-R-phycoerythrin. Labelled target sequences are transferred for analysis into a QIAGEN LiquiChip 200 workstation. Although not optimised performance efficiency of this device compared to conventional procedures was just slightly reduced and shows potential for improvement.

By changing the sample, reagents and especially the primers other applications can be realized by analyzing other pathogens.

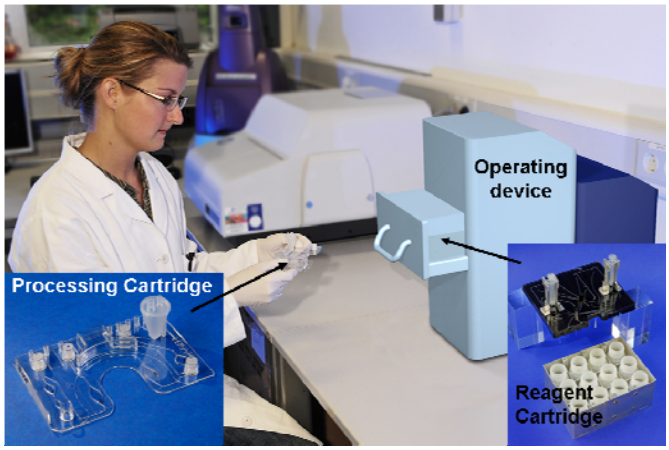

Fig. 5 Lab-on-a-chip system for the automated analysis of pathogens in nasopharyngeal swab samples. The device is composed of the operating device bearing all components for process control, an injection moulded disposable processing cartridge comprising structures for sample lysis, SPE, RT-PCR, nested PCR and target labelling and a reagent cartridge for 24 analyses.

SUMMARY AND CONCLUSION

The presented microfluidic-based platforms address actual challenges in biotechnology and health care envisioning facilitated, fast, thereby robust and reliable point-of-care systems. Miniaturisation, integration and automation promote time- and labour-saving leading to faster overall analysis times in diagnosis and therapy monitoring. The generic character makes these platforms fast and low-cost tools for a diversity of applications that require clinical diagnostics at the point-of-care.

Parallel development of disposable analysis chip and corresponding instrument lead to cost-efficient LoC-systems suitabile for serial production. One major bottleneck for LoC systems still is robustness at affordable costs. To achieve this demand, simplicity of assay and instrument is the key strategy for future integrated molecular diagnostic LoC devices[8]. Once established, these systems bear the possibility to get adapted to other applications thereby open completely new markets for in-vitro diagnostics.

By growing understanding of the mechanisms and strategies of pathogens and diseases, simplifying assays and instruments further by applying the growing knowledge in transferring processes into microsystems, and simplifying and optimizing production technologies the future vision of small, simple, non-invasive and generic analyzers moves closer.

ACKNOWLEDGMENTS

The research leading to these results has received funding from the European Community's Sixth Framework Program (IST-NMT-CT-2005-017319, FP6-2004-IST-026752), the German Bundeswehr and the German BMBF (FZK 16SV3318).

Thanks to all the staffs of IMM, especially from the Fluidic and Simulation department, and the project partners who did all the work resulting in the systems presented here.

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