Investigations of paper-based lab-on-chips for on-diaper point-of-care screening of urinary tract infections

by
Shilun Feng

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Department of Micro and Nano Systems Technology
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Thesis Description

The thesis focuses on investigations of MEMS-based solutions to obtain easier urinary tract infections (UTI) diagnoses. For elderly patients, collecting a sufficient amount of urine for UTI tests is usually a painful and time-consuming procedure. Diapers are often employed in these patients, and a diaper might become a sampling tool for urine. A diaper-based UTI test would be a convenient solution, but to date, few diagnostic tools have been designed to test urine samples from diapers. This thesis strives to develop a point-of-care real-time 'on diaper' detector that would allow nursing staff to easily distinguish UTI patients. As a student with a biomedical background, my tasks included developing a sampling module for urine collection from diapers; investigating the biomarkers and methods used to detect UTIs; execution and testing the successful point-of-diagnostics biosensor for the early on-diaper screening of UTIs in the elderly.

The thesis comprises of 4 parts:

Part 1 presents an introduction to the designs and its execution.

Part 2 provides the background and describes the investigations on the point-of-care diagnostics, UTIs, the methods used to detect UTIs, biomarker detection, lab-on-chip technology and biomedical microfluidics. These topics are included in this background section because they are related to the content presented in later chapters. The contents are related with the topic of my thesis which is a point-of-care diagnostics biosensor for the screening of UTIs in elderly people. A specialized background is also presented in each chapter.

Part 3 describes the preparations and trial methods. Sampling modules and cell cultures were studied in the bio-MEMS lab. Of course, there are so many methods tried in the project, however, two methods were totally tried by me and the final method which is paper-based lab-on-chip method is also partly tried by me to prove the feasibility of detecting UTIs.

In the first stage, lab-on-chip bioluminescent detection method was attempted by combining the structure of an existing living-cell chip, which was first exploited by Dr. Xinyan Zhao, with a novel biological protocol and operation process to elucidate the point-of-diagnostic for UTIs to provide a highly efficient long-term continuous analysis platform for measuring the concentration of adenosine 5'-triphosphate (ATP) and thus detecting UTIs based on bioluminescence. The chip that was introduced in 2012 is no longer available for living cells because the operation process, chemical reactions and aims used to detect UTIs have changed. There are some disadvantages of LOC: its complicated operations require sample pre-preparation, and the entire system is much more complicated. More importantly, we are using ATP as the biomarker, which was not sensitive or quantitative for UTI detection; specialised biomarkers should be used for the quantitative detection of UTIs. Here I published 2 conference papers based on these contents and used part of the contents directly inside the

thesis, one paper is titled "Design and Characterization of a Lab-on-chip for Continuous Bioluminescent Measurements of ATP," which was the publication for 2014 IEEE International Symposium on Medical, Measurements and Applications (IEEE MeMeA 2014), Lisbon, Portugal, June 11-12 2014 and another paper is titled " Detection of Urinary Tract Infections on lab-on-chip device by measuring bioluminescent photons emitted from ATP," which was one of Conference Proceedings of the 36th Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC'14), Chicago, USA, 2014.

Luckily, a commercial nitrite electrochemical sensor was available in the lab, so I attempted to build a relationship between the numbers of *E. coli* and nitrite ion concentration reflected by the measuring value shown on the sensor screen, which makes it possible to detect possibilities of UTIs quantitatively. Many experiments were conducted in the bioMEMS lab, and I had proved that specialized biomarkers can be used for the quantitative detection of UTIs. The electrochemical detection method is simple, has the ability to detect on diapers, does not require sample pre-treatment, is portable and micro-assembled, and can be used for multiple detections (though it must be cleaned between uses); however, the sensitivity of the commercial electrode is easily affected by noise. This electrode is also challenging to use when it is in direct contact with the diaper. Most importantly, we need to detect multi-biomarkers other than nitrite to obtain the most reliable result, so multi-selective electrodes must be used in the process design, which requires time for their design and fabrication. Unfortunately, a touch sensor takes 2 years to develop, which was too long for this study; however, if we had sufficient time and money, it is still a good technique to develop. Here I also published two paper and I used part of the contents, one of these is named as " Applied Technology in Diaper-based UTI Testing for Elder People by Using Nitrite Ion Selective Electrode," which was published in 2014 2nd International Conference on Mechanical Engineering, Civil Engineering and Material Engineering (MECEM 2014) and another is named as "Quantitative detection of *Escherichia coli* and measurement of urinary tract infection diagnosis possibility by use of a portable, handheld sensor" for 2015 IEEE International Symposium on Medical, Measurements and Applications (IEEE MeMeA 2015), Torino, Italy, May 07-09, 2015.

Part 4 focuses on the main topic: the investigation and excutation of paper-based lab-on-chip for the point-of-diagnostic of UTIs that combines the lab-on-chip and biomarker quantitative detections with the help of lateral flow capillary force. The convenience of this device meets my aims for collecting urine on diapers and analysing the results both easily and accurately: distribute the urine sample into multiple segregated regions for multi-parameter detection; move the urine sample to the pads without the help of a pump; be compatible with small volumes of urine sample; be economic; be disposed of by incineration; and keep the results constant for a long period of time. Investigations of paper, lateral flow, paper-based microfludics, fabrication methods and theorical studies were done, one design which makes

channels on paper was made by me and simple tests were done for this conception, which seemed feasible. At the continuous step, I turn to work with Dr. Nuno pires, Dr. Zhong Fang and master student Chaohao Chen and Jennifer Panugan, their design was to make the hydrophilic channel to transport the sample on the paper and hydrophobic barriers to form the channel, wax-based fabrication method was investigated and tried by me in the bioMEMS lab and simple tests were also done by me. I also visited the factories who can make the paper-based assays which could also be helpful for realising the final product. The on-diaper UTI diagnostic could be completed with reliable results and convenience. Continuous tests of this reaction layer of biosensor were performed in the bioMEMS lab, fold generated by wearing will not affect the result, the channels were formed ideally by hydrophilic wax and the results were kept for more than 24 hours, which covers the disadvantage of the commonly used urine strips. Additionally, it only took 2 min to read the results, which covers the disadvantage of both the gold method-urine culture and microscope. It proved to be a successful point-of-diagnostics biosensor for the diaper-based screening of urinary tract infections (UTIs) in elderly people.

Further work will be done to fabricate the product precisely and medical examination will be done for its safety; moreover, cell phone or holdheld sensor will be used to read out the quantitative results rather than the basic yes-or-no answer. So many works had been done during my master theis, I hope this promising and useful point-of-care diagnostics biosensor will be the successful one for diaper-based screening of urinary tract infections (UTIs) in elderly people.

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Shilun Feng, Bio-MEMS

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Supervisor: Tao Dong

Abstract

UTIs are common infection diseases in the hospitals, especially in the elderly. Detecting UTIs in the elderly is a difficult and time-consuming procedure. Sampling and detection on a diaper could be the solution.

Sampling from diapers has been investigated and experimented, and the results have shown that injecting salt water can release the urine absorbed by the diaper. Different biomarkers have been investigated, and two methods were attempted using the existing tools: a bioluminescence ATP-detection lab-on-chip and an electrochemical sensor for the diagnosis of UTIs after extracting urine from the diaper. Finally, based on the literature, bio-sensing paper-based lab-on-chip were used for UTI point-of-diagnosis. By using a combination of lateral flow and paper-based lab-on-chip, a urine sample can be conveniently collected and analysed directly on the diaper.

For the bioluminescent lab-on-chip method, an existing novel multifunctional silicon lab-on-chip (LOC) was used to provide a highly efficient long-term continuous analysis platform for measuring the concentration of adenosine 5'-triphosphate (ATP) and thus detect the UTI based on bioluminescence. A new two-sided silicon lab-on-chip (LOC) was also designed, and numerous investigations proved that combing it with segmented flow analysis (SFA), T-junction droplet generator, which is stable to transport the sample, and a micro mixer can achieve an outstanding mixing efficiency in a small space. The existing chip had been tested, and the materials were well prepared before testing the PMT detecting system. The data from PMT were visualized by the Labview™, and they showed good linearity between the voltage values and the ATP concentrations, which ranged from 2×10^{-12} M to 2×10^{-8} M. Fresh urine samples with different amounts of *Escherichia coli* were measured by the system and showed a good linearity trend between the voltage values and the number of *E. coli*. This study successfully expressed the concept of measuring ATP directly in the urine to quickly and accurately detect UTI on a microfluidic chip.

An electrochemical nitrite sensor was assembled and calibrated, and the artificial urine sample was measured; the feasibility of the electrochemical nitrite sensor, including the error effect, was detected around -5.1~2.3%. The possibility of detecting artificial UTIs in urine samples is approximately 95.5%, and the approximate relationship between the number of *E. coli* and the electrode potential was E=228.3193-3.78225×L n (N+2.29101e6), thus building the relationship between UTI

possibility and measurement. Finally, the electrochemical sensor array was conceived and designed to measure different biomarkers and thus obtain the maximum UTI possibility and to show the data for the UTI possibility directly on the screen. Furthermore, this array can easily be used and transported for home-users or patients in hospitals.

The bio-sensing paper-based lab-on-chip method for determining UTIs with an on-diaper point-of-care diagnosis application combines the lateral flow and biochemistry reactions. Topics related with paper-based lab-on-chip microfludics and fabrication methods were investigated. The design to make channels on paper and tested were done by me. Another design for realising the reaction on one paper with reactions to form channels and barriers were designed by others but the wax-based fabrication method for this were investigated and finally fabricated by me. Continuous tests of this reaction layer of biosensor were performed in the bioMEMS lab, fold generated by wearing will not affect the result, the channels were formed ideally by hydrophilic wax and the results were kept for more than 24 hours, which covers the disadvantage of the commonly used urine strips. Additionally, it only took 2 min to read the results, which covers the disadvantage of both the gold method-urine culture and microscope. It proved to be a successful point-of-diagnostics biosensor for the diaper-based screening of urinary tract infections (UTIs) in elderly people.

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Chapter 1

Introduction

UTIs are among the most prevalent infectious diseases in the general population [1]. Moreover, UTI is a common reason for hospital admission in patients aged 75 and older[2]. It is challenging for frail elderly people to detect the cases of symptomatic UTI with an over diagnose tendency[3]. Urine is a good resource for biomedical monitoring because it is full of biomarkers, easily obtained in a non-invasive manner, simple to handle and an economic resource. Collecting enough urine for urinary tract infection (UTI) tests is often a painful and time-consuming procedure in elderly patients. This thesis focuses on providing a MEMS-based solution for more easily diagnosing a UTI. Diapers are often utilized with UTI patients; therefore, the diaper is a potential urine sampling location. The diaper-based UTI test will be a convenient solution, but there are few diagnosis tools that were designed to test urine samples on diapers. A UTI can be detected in samples obtained from a diaper, and the nurse would not be concerned with extracting urine from the patients (elderly or disabled people).

The medical characteristics of a UTI and methods for sampling from the diaper must be investigated to design novel methodology. Different methods and biomarkers have been investigated, and bioluminescence ATP-based detection on a chip and electrochemical sensors were studied as UTI diagnostics after extracting urine from a diaper with existing tools. Finally, based on these two methods, a paper-based biosensor was fabricated and tested; this tool is much easier to utilize, and it can be applied on-diaper for the point-of-care (POC) diagnostic detection of a UTI.

The first problem related to urine extraction from the diaper and the effectiveness of urine for UTI detection. Some experiments were performed using normal diapers. Sampling from the diaper was investigated, and injecting salt water released the urine that had been absorbed by the diaper.

Because urine samples from UTI patients are difficult to obtain, another problem related to making artificial urine for laboratory research. E. coli is the cause of 80–85% of UTIs [4]; therefore, E. coli was cultured in the lab, and artificial urine was created by adding different amounts of E. coli to fresh urine.

The third problem related to choosing a diagnostic for a UTI. A UTI is generally diagnosed using urinalysis reagent strips followed by cell culture and microscopy analysis [5]. The shortcomings of this methods include poor patient interactions while

obtaining urine samples (especially in patients with incontinence or cognitive impairment), sample contamination and the time required to complete the analysis (2-3 days) [6]. Moreover, the high prevalence of bacteriuria in elderly individuals makes it difficult to determine whether a new symptom is related to bacteria in the urine [7]. In this thesis, two methods were evaluated using existing tools, including bioluminescence-based ATP detection using a lab-on-chip (LOC) and an electrochemical sensor for diagnosing a UTI after extracting urine from a diaper. Finally, based on the experiments, a biosensing paper-based LOC for the POC diagnostics of a UTI was evaluated. With the combination of lateral flow applications and paper-based LOC, the urine sample can be collected and analysed directly on-diaper in a convenient manner.

For the bioluminescent LOC method, a novel multifunctional silicon LOC was designed and fabricated by Dr. Xinyan Zhao from HBV as an environmental live cell chip; this LOC was applied with my new biological protocol to provide a highly efficient, long-term continuous analysis platform for measuring the concentration of adenosine 5'-triphosphate (ATP) to detect a UTI based on bioluminescence. The multifunctional microfluidics-based biochip is promising. It provides exciting possibilities for parallel immunoassays, high-throughput sequencing, DNA sequencing, blood chemistry for clinical diagnostics, and environmental toxicity monitoring. The complexity of future microfluidic devices will be significant due to the need for multiple and concurrent biochemical assays on reconfigurable and multifunctional platforms [8].

Rapid detections of cells in drinking water and food are of great importance to public hygiene and food safety. The bioluminescent assay method, using an ATP dependent-luciferin-luciferase reaction, is a reliable technique to measure biomolecules [9] in a sample or the number of cells [10,11]. And urinary tract infection, as the most common organ system to experience bacterial infections has also being using the ATP Bioluminescent Measurements [11]. The mechanism of the bioluminescent reaction occurs as follows (equation 1) [12]:

$$ATP + luciferin + O2 + luciferase \rightarrow AMP + oxyluciferin + CO2 + PPi + hv \quad (1)$$

Luciferase produces cold light (~560 nm) by the ATP- dependent oxidation of d-luciferin. Consequentially, the amount of ATP is directly associated with the amount of emitted light by luciferase, which in turn represents the amount of cells in the solution. Despite this, the poor stability and high cost of luciferase and d-luciferin in bioluminescent reactions prevent the extensive usage.

A promising alternative is to manipulate liquids as discrete droplets based on the principle of continuous fluid flow [13]. Segmented Flow Analysis (SFA) uses

nitrogen or air bubbles to separate a sample into short fragments and then manipulates the liquid as discrete segments. This method can make the process automatic and stable, which can be helpful for online continuous systems, by controlling the time and velocity of the samples and reagents that are travelling in the chip. Specifically, it can also guarantee sufficient oxygen supply for the cells in droplets. Because there are some disadvantages of LOC, such as numerous processes, complicated operations, and the need for sample preparation, the whole system is very complicated and is not suitable for one-time use. More importantly, we are using ATP, which is neither sensitive nor quantitative; specialized biomarkers for quantitatively detecting a UTI should be identified.

An existing electrochemical nitrite sensor was used to quantitatively detect the nitrite concentration in urine, and by building the approximate relationship between the nitrite concentration and number of *E. coli*, the electrochemical nitrite sensor can count the number of *E. coli* and diagnose a UTI. Since the 1920s, when the urinary nitrite test was first developed, the dipstick assay has been an important component of modern UTI diagnostics. Bacterial growth in slightly acidic urine is inhibited if exogenous nitrite is added [14, 15]. This inhibition is potentiated in the presence of the reducing agent ascorbic acid. Indeed, the acidification of urine has been used in traditional medicine to prevent and treat UTIs [16]; however, clinical trials have not provided sufficient convincing findings for this concept. In this method, we have expanded the role of nitrite beyond the diagnosis of an infection and have utilized nitrite in the new electrochemical urinalysis system. The urinalysis system for home users has aroused the general interest of researchers because urine tests are also suitable for household daily monitoring of personal health conditions. The strips are too simple to fulfil the demands of home users. These users are required to handle their urine samples and the commercial strips step by step; moreover, the results on the strips must be evaluated by the home users themselves according to the user manual, and the results change markedly over time. Many home users are confused by the strip tests.

In my artical, a novel automatic urinalysis system that uses an electrochemical sensor to quantitatively detect nitrite is proposed to provide personal urinalysis services for home users. The system will involve multiple functions, including automatic sampling, rapid detection, and auto-decontamination. Moreover, this system can be easily used and can analyse and report the data suggesting the possibility of a UTI directly on the screen. Home users can monitor their results using personalized health information technology (HIT); this is one of the few urinalysis systems with a built-in connection to IT technology, and it suggests that the abundant medical information in urine samples could be measured and exported directly into a

web-based medical database, thus providing a low-cost solution for automatic urinalysis services for patients in hospitals and at home.

The method of biosensing paper-based LOC for on-diaper UTI diagnostics, which is easy to implement and utilize, combines lateral flow applications and microfluidic paper-based analytical devices (μPAD) to create a LOC for quantitative biomarker detection. The convenience of this device meets my aims of collecting urine on diapers and analysing the results both easily and accurately: the ability to distribute the urine sample into multiple segregated regions for multi-parameter detection and to move the urine sample to the pads without the help of a pump; compatibility with small urine sample volumes; economical; disposed of by incineration; and consistent results over a long period of time. The on-diaper UTI diagnostic was created with reliable results and convenience. Investigations on the paper, lateral flow assays, paper-based biomedical microfludics and theorical studies were done, simple experiments on normal strips were done, which proves it is important to keep the pads covered and wet to make the results validated for a long duration and it is important for the biomarkers fabricated separately to avoid the cross-talk. One design of making channels on paper to reach the aim was done by me and fabrication methods about this were investigated, after that, simple experiments had been done to prove the feasibility. A novel and more promising design of paper-based lab-on-chip was done by Dr. Nuno Pires and so on, so I turned to focus on the fabricating their design which is using the hydrophilic barriers to form the hydrophobic channel for the biological samples forced by capillary forces. Wax-based fabrication methods were investigated and tried by me and the simple tests for the final product was also done, which proves generated fold by wearing will not effect the results, the barriers is good enough to help for forming the channel and the results could be kept for more than 12 hours, which eliminated the disadvantage of normal urine strips; it only took 2 minutes to read the result, thereby eliminating the disadvantages of both the gold standard urine culture and microscopy. This novel method was demonstrated to be a successful POC diagnostics biosensor for diaper-based screening for UTIs in elderly people.

Chapter 2

Background

In this chapter, I described the investigations of POC diagnostics, UTIs, the methods used to detect UTIs, detection biomarkers, LOC technology and medical biomedical microfluidics. These are listed in the background section because they are related to the content presented in later chapters. The topic of my thesis is a widely desired and promising POC diagnostics biosensor for UTI screening in elderly people. Specialized background is also provided in each chapter.

2.1 Point-of-care diagnostics

The clinical POC is where clinicians deliver healthcare services and products to populations at the time of care. Diagnostics are commonly used to diagnose the cause of a patient's symptoms, to monitor treatment efficacy and to screen for potential diseases in asymptomatic but high-risk patients. If a rapid test kit is affordable, simple to operate, and accurate at diagnosing, it could be a lifesaver. Thus, diagnostics are of great importance in the healthcare system, and they have a critical effect on epidemiological and clinical decision-making. To meet the needs of the developed world's medical community, biomedical engineers had developed several traditional technologies. However, diagnoses are often based on clinical experience and lack laboratory evidence in resource-limited settings. Therefore, a major challenge is to develop diagnostic tests that can meet the needs of people in the developing world. Compared with standard laboratory testing, POC diagnostics are rapid, simple and inexpensive and are thus suitable for resource-limited settings. Therefore, POC diagnostics are increasingly essential for initiating and scaling on-site medical care for the prevention and control of infectious diseases. The WHO has set seven guidelines for the development of diagnostics in resource-poor settings. These tests must be: (i) affordable, (ii) sensitive, (iii) specific, (iv) user-friendly, (v) rapid and robust, (vi) equipment-free, and (vii) delivered to those who need it, corresponding to the acronym[17].

2.2 Urinary tract infections

The urinary tract is the most common organ system to experience bacterial infections [18].UTIs are the most common infections in both hospitalized and community

patients, [19] which are common infects causing serious morbidity and significant expenditures in healthcare dollars and lost wages[20]

UTIs due to Staphylococcus aureus typically occur secondary to blood-borne infections. E. coli is the cause of 80–85% of UTI[4] Staphylococcus saprophyticus being the second cause with 5–10%. [4] Rarely they may be due to viral or fungal infections.[21] Other bacterial causes include: Klebsiella, Proteus, Pseudomonas, and Enterobacter. These are uncommon and typically related to abnormalities of the urinary system or urinary catheterization. [22]

Urine is helpful for urinary tract disease assessment and for the diagnosis and treatment of diseases of the kidney or certain metabolic or systemic diseases such as diabetes and autoimmune and endocrine diseases. The components in the urine for 24 hours are varied, and first-morning urine is usually the best specimen. Normally, midstream urine is used because urinary bacteria or cells can reduce pollution and because this urine is suitable for bacterial culture. The test's sensitivity depends on the intended patient group, and *Staphylococcus saprophyticus* is not relevant in the elderly. A properly collected clean-catch of midstream urine after cleansing of the urethral meatus is adequate for complete urinalysis.

Another important factor is the interval of time that elapses from collection to examination in the laboratory. Changes that occur with time after collection include 1) decreased clarity due to solute crystallization, 2) increasing pH, 3) loss of ketone bodies, 4) loss of bilirubin, 5) dissolution of cells and casts, and 6) overgrowth of contaminating microorganisms. Generally, urinalysis may not reflect the findings of absolutely fresh urine if the sample is over 1 hour old. Therefore, it is important to get the urine to the laboratory as quickly as possible, more experiments would be done to examine this chapter in the latter chapter.

There are some difficulties to detect UTI for the elder people, one is there will be Asymptomatic bacteriuria (ASB) with no symptoms; the second is Gram-negative coliforms reduce urinary nitrates to nitrites if there is sufficient nitrate in the patient's diet and the urine has been in the bladder for at least 4 hours, but this test will fail to detect atypical organisms (e.g., Gram- positive organisms and Pseudomonas) [23], which are more common in elderly people, the last is the common drawback for obtaining urine samples and sample contamination.

2.3 Methods to detect UTI

In straightforward cases, further laboratory confirmations are not needed after a diagnosis made and treatment given based on symptoms. In complicated or questionable cases, it may be useful to confirm the diagnosis via urinalysis, looking

for the presence of urinary nitrites, white blood cells (leukocytes), or leukocyte esterase. Another test, urine microscopy, looks for the presence of red blood cells, white blood cells, or bacteria. Urine culture is deemed positive if it shows a bacterial colony count of greater than or equal to 10^3 colony-forming units per mL of a typical urinary tract organism. Antibiotic sensitivity can also be tested with these cultures, making them useful in the selection of antibiotic treatment. As symptoms can be vague and without reliable tests for UTIs, diagnosis can be difficult in the elderly people.

There are some Quick Methods to detect UTIs, which are Microscopic Methods-Gram stain, Enzymatic Methods (Catalase test、Glucose oxidase、Reagent Strip Testing-Nitrate reductase (Greiss) test, Leukocyte esterase 、 Protein 、 Haemaglobin)，Colorimetric Filtration，Bioluminescence，Photometry Detection of Growth， Electrochemical detection method， Microcalorimetry， Photometry， Turbidimetric screening，Limulus Amoebocyte Lysate Endotoxin Test，Malthus system[24].

Early diagnosis of UTIs was mainly by urinalysis reagent strip and/or microscopy which can also determine the performance of urine cultures. The objective of this work was to evaluate the sensitivity, specificity and sensitivity of urine [25]. In a meta-analysis of studies performing urine dipsticks on older patients between 1990 and 1999, the sensitivity and specificity were calculated as 82% and 71%, respectively, for nitrites or LE positivity [26]. However, a study has suggested that urine dipsticks given the high prevalence of ASB and pyuria, the percentage of suspected UTI that could be excluded was small (those tested accounting for 12%, all suspected UTI accounting for 7%) [27]. Even urine dipstick test has a very poor sensitivity when performed on randomly selected urine specimens[28].Further difficulties with this method include obtaining urine samples (especially in patients with incontinence or cognitive impairment) and sample contamination. The traditional basis for evaluating urinary tract pathogens (uropathogens) is antibiotic susceptibility testing and urine culture, The major drawback of the current microbiology approach is the time lapse of 2 to 3 days between specimen collection and the availability of objective evidence for treatment selection. Several days may pass before a definitive diagnosis is made and an objective treatment strategy can be implemented.

Similarly, the high prevalence of ASB means that urine culture results alone cannot be used to diagnose clinically relevant UTI in elderly people. Moreover, the high prevalence of bacteriuria in elderly individuals makes it difficult to know if a new symptom is related to bacteria in the urine. There are different views concerning this relationship and bacteriuria often leads to antibiotic treatments[29].

2.4 Biomarkers detection

2.4.1 ATP

Use of bioluminescence as a urine screen was first de- scribed in 1944 [30]. Application of the luciferase enzyme and luciferin to the detection and quantitation of bacteria was originally described by Chappelle and Levin, who used a luminometer to measure bioluminescence (as relative light units [RLU]) [31]. Firefly lantern bioluminescence is produced by a biochemical reaction in which the luciferase enzyme utilizes ATP in the oxidation of luciferin to adenyl-oxyluciferin, with the concurrent release of photons in proportion to the amount of ATP that is converted to AMP. The mechanism of the bioluminescent reaction occurs [32] as equation (1).

Luciferase produces cold light (~560 nm) by the ATP- dependent oxidation of d-luciferin. Consequentially, the amount of ATP is directly associated with the amount of emitted light by luciferase, which in turn represents the amount of cells in the solution.

Although an incubation step is required, urinary ATP concentration luminance analysis method can provide the rapid and reliable result. Several problems including the presence in urine of free nonbacterial ATP, the intracellular ATP contained in somatic cells, and luciferase inhibitory substances and the variation in ATP content among bacterial species appears in initial attempts to apply this methodology to the enumeration of bacteria in clinical urine specimens, all of which compromised the quantitative aspect of the assay [33]. These problems were efficiently solved by using buffers and reagents for the release and destruction of nonbacterial ATP in clinical urine specimens. The reported threshold for positivity was 10^5 CFU/ml, with a sensitivity of 86 to 95%, a specificity of 75 to 82%, a positive predictive value of 36 to 77%, and a negative predictive value of 88 to 95% [34]. It is reported that urinary ATP concentration analysis is useful for determining UTIs and renal damage caused by drugs and by means of the firefly luciferin-luciferase method, reference value was established as 1.77×10^{-10} M to approximately 7.70×10^{-9} M. [35]

Another paper reported that using the microfluidic device with ATP standard solutions, the bioluminescence intensity was linearly correlated with 2×10^{-12} M to 2×10^{-8} M of ATP [36] , which was more accurate, however, it was not applied for detecting UTI. Thereby, in this paper, I combine the usage of microfluidic device to detect the ATP as assay, it can not only quickly get the accurate result ranging from 2×10^{-12} M to 2×10^{-8} M but also prevent the poor stability and high cost of luciferase and d-luciferin in bioluminescent reactions .

2.4.2 E.coli and other bacteria

UTIs are among the most common bacterial infections in humans, the majority of bacterial strains including *E.coli* which is the governing causative species[37], *Klebsiella, Proteus, enterobacter* causing UTI have a nitrate-reducing capacity to produce the nitrite. Nitrite is known to give a sensitivity on 35% to 85%, but highly specific [38].

The test for nitrites is a rapid screening method for any possible asymptomatic infections caused by nitrate-reducing bacteria. Some of the gram-negative bacteria species that most commonly cause urinary tract infections (*Escherichia coli, Enterobacter, Klebsiella, Citrobacter and Proteus*) have enzymes that reduce the conversion of nitrate in urine to nitrite. The test is a rapid screen for possible infections by enteric bacteria, but it does not replace the urinalysis tests or microscopic examination as diagnostic tools or as subsequent monitoring because many other microorganisms that do not reduce nitrate (gram positive bacteria and yeasts) can also cause urinary infections.

Microbial organisms that are found in all but the most scrupulously collected urine should be interpreted in concert with clinical symptoms. A diagnosis of bacteriuria in a case of suspected UTIs requires culture analysis. A colony count may also be performed to determine whether significant numbers of bacteria are present. Generally, more than 100,000/ml of one organism reflects significant bacteriuria. The presence of multiple organisms reflects contamination. However, the presence of any organism in catheterized or suprapubic tap specimens should be considered significant.

2.4.3 Nitrite

Urinary nitrite has been used as an indicator of UTIs for many years. Nitrate is reduced to nitrite by the enzyme nitrate reductase, which is present in gram-negative bacilli, and causes urinary tract infection. If we know the concentration of nitrite quantitatively, then we know the possibility of a UTIs. It has been suggested that testing be performed only on urine that has remained in the bladder overnight or for at least 4 h to allow for a higher concentration of urinary nitrite [39].

Indeed, at a basal urinary pH and even in slightly acidic urine, the bacterial growth was very good. In contrast, the growth of *E. coli*, *P. aeruginosa* and *S. saprophyticus* was dose-dependently inhibited by nitrite in acidified urine. The inhibitory effect of nitrite was greater at lower pH. The addition of ascorbic acid (10 mM) further enhanced the inhibition of bacterial growth by nitrite. Increasing concentrations of nitrite (0-500 μM) with a fixed concentration of ascorbic acid (10 mM) caused a dose-dependent inhibition of *E. coli* growth. The higher the nitrite concentration is, the stronger inhibition will be. However, if bacteria were pre-

incubated in basal urine without the addition of sodium nitrite, no inhibition was noted when the culture was transferred to the acidic urine. This result indicates that a normal diet in a patient treated with urinary acidification is likely insufficient to excrete a sufficient amount of nitrates in the urine to achieve antibacterial effects [40]. Moreover, nitrate was found to be stable in urine and plasma samples when they were incubated for 2 h at 37℃ or stored for 24 h at 4℃. [41] Gram-negative coliforms reduce urinary nitrates to nitrites if there is sufficient nitrate in the patient's diet and if the urine has been in the bladder for at least 4 hours[42].

2.4.4 LE

Leukocyte esterase (LE) is a urine test to determine the presence of white blood cells and other abnormalities that are associated with infection.Pyuria, or the presence of WBC's in urine, has long been established as a UTI marker. [43]Pyuria is typically determined by urine microscopy and reported as the number of WBC's per high power field (WBC/HPF), which requires an experienced laboratory technician counting the WBC under the microscope using the 40×objective. Pyuria may also be indirectly determined as part of the urine dipstick to measure the enzymatic activity of another WBC-derived protein, leukocyte esterase (LE).

In the 1950s, dry chemistry strips tests were the only method for detecting UTIs. Braga et al. proposed the reagent strip with leukocyte esterase that was designed for urine testing [44]. A urine sample that tests positive for both nitrite and leukocyte esterase should be cultured for pathogenic bacteria. In 1970, scholars detected urine specimens by using human blood or bone marrow leukocytes [45].

European countries reported using early LE strips to detect white blood cells in the urine continuously [46]. The enzyme is not present in the normal serum or urine or upon inflammation of the kidney tissue [47].

The urine test strip reaction is based on the action of leukocyte esterase in catalysing the hydrolysis of an ester of indolecarboxylic acid. The indoxyl that is liberated combines with a diazonium salt to produce a violet-coloured azole dye.

2.4.5 LF

We hypothesize that urinary lactoferrin (LTF), a 80 kDa iron-binding protein that is secreted by polymorphonuclear white blood cells (WBC), is both a useful target for electrochemical immunoassay development and a predictive UTI biomarker because the average concentration of LTF was found to be 30.4 ng/ml in healthy urine and 3300 ng/ml in infected urine. [48] We correlated the urinary LTF level with the presence of WBC in urine (pyuria)—an important hallmark of UTIs. As one of the proteins secreted by the WBCs, urinary lactoferrin (LTF) has antimicrobial properties and competes with pathogens for iron, which is important for pathogen survival [48].

2.4.6 Urine sediment

In a sample of urine sediment from a patient suffering from a urinary infection, it is possible to observe leukocytes (small, round and granular), erythrocytes (small, round and biconcave) and epithelial cells (large and polyhedral). Urinalysis can reveal diseases that have gone unnoticed because they do not produce striking signs or symptoms. The sediment is first examined under low power to identify most of the crystals, casts, squamous cells, and other large objects. The numbers of casts observed are usually reported as the number of each type found per low power field (LPF).

Few white blood cells (see microscopic examination) are present in urine, and this test is thus negative. Pyuria, or the presence of WBCs in urine, has long been established as a UTI marker. Pyuria is typically determined by urine microscopy and is reported as the number of WBCs per high power field (WBC/HPF), which requires an experienced laboratory technician to count the WBCs under a microscope using the 40× objective. When the number of WBCs in urine increases significantly, this screening test is positive. If two or more leukocytes per each high power field appear in non-contaminated urine, the specimen is likely abnormal.

The presence of Red Blood Cells indicates upper and lower UTIs, nephrotoxins, and physical stress. Theoretically, no red cells should be found, though some do find their way into the urine, even in very healthy individuals. However, if one or more red cells can be found in every high power field and if contamination can be ruled out, the specimen is probably abnormal.

Moreover, because of the differences observed in the results that were obtained using eye detection of the visible colour level, the automatic analysis of urine has been developed worldwide.

2.5 lab-on-chip technique

A LOC is the integration of one or more functional laboratory units on one chip; the size of the unit ranges from micrometres to micrometres, and the processes can be accomplished on one chip.

Although the application of LOCs is still novel and modest, more companies and applied research groups are attempting to utilize LOCs in different fields, including analysis (e.g., chemical analysis, medical diagnostics and environmental monitoring) and synthetic chemistry (e.g., rapid screening and microreactors for pharmaceutics).

LOCs have several merits, including reagent consumption, a quick response time and a reduced reaction time, because of the short diffusion distances and high surface-to-volume ratios. Due to the integration of their functional units, the whole process or multiple processes can be finished automatically in the limited area; LOCs can also

be fabricated in batches, thereby providing cheap portable platforms for chemical and biological studies.

Some disadvantages exist as well. The technology has not been fully developed because of the novelty and physical and chemical effects, such as capillary forces, surface roughness, and chemical interactions of construction materials during the reaction processes, which become more dominant on a small scale. For these reasons, the processes used in LOCs are often more complex than those implemented using conventional lab equipment.

Cost, scalability, and recyclability should be considered when choosing materials and fabrication techniques. Photolithography is the commonly utilized method for fabricating most LOCs. Silicon materials are always chosen because of the well-developed fabrication processes and techniques. To meet other requirements, additional novel and promising fabrication methods have been quickly developed, including those involving glass, ceramic and metal etching; deposition and bonding; polydimethylsiloxane (PDMS) processing (e.g., soft lithography); thick film; and stereolithography as well as fast replication methods via electroplating, injection moulding and embossing, especially on paper or textiles.

2.6 Biomedical Microfludics

Because of the merits of biomedical microfluidics, this technique has become more popular in developing countries [49]. Its merits include the ability to be combined with functional units (such as pumps and valves) to create a complete analytical system that mimics an actual laboratory setup [50] but requires only small reagent volumes to operate and can function in an automated fashion [51]. Thereby, it can economize the use of reagents, save money, reduce the complexity of the processes and finish the test in a short time in a controlled manner without compromising sensitivity and accuracy [52]. Most microfluidics platforms use materials such as glass, silicon, and polymers, such as PDMS and polymethylmethacrylate (PMMA) [53], that require complicated fabrication and external equipment to connect with. Because of these two drawbacks, these platforms are not available for development in resource-limited countries [54]. Therefore, paper-based LOCs will be more popular because they can avoid the abovementioned drawbacks of a complex process and the need to be combined with external equipment for operation. Moreover, it is easy to develop portable and cheap paper-based LOCs. In the past 2 decades, biomedical microfluidics platforms have developed from a clean room product to a commercial product and have been demonstrated to be suitable for POC diagnostics; for these reasons, paper-based LOC biomedical microfluidics platforms have potential in developing countries [54].

In this chapter, POC diagnostics, UTIs, methods to detect UTIs, and biomarker detection are introduced and discussed. This section provides a brief academic foundation for the later research.

Chapter 3

Preparation and EXPERIMENTAL methods

After the investigations and noting that collecting samples from elderly people was painful and unreliable, we attempted to extract urine from used diapers. The diaper sampling module was investigated in the lab; injecting a salt solution could release the absorbed urine. Cell culture was performed and served as the basis for every method because the number of *E. coli* indicates the possibility of a UTI; if the number is greater than 10^5, the patient can be said to have a UTI. The cultured urine was tested using an available silicon-based chip that was developed and designed by Dr. Xinyan Zhao from HBV for environmental live cell detection. I completely changed the biological protocol and operation process and took advantage of the chip structure to ultimately successfully detect a UTI by measuring the ATP concentration. I must emphasis that I learned how to design, work with and draw the structure of the chip. I designed one chip, drew a two-sided silicon LOC using SolidWorks by myself, and examined the mixers, reaction channels and other structures, which provided the foundation for me to develop a paper-based LOC. After elucidating the advantages and disadvantages of this method, I chose to study a nitrite electrochemical ion sensor because it is more specialized; I hoped that the use of this sensor would enable the rapid quantitative detection of a UTI with great consistency. With an available tool in the BioMEMS lab, the nitrite solutions and the cultured *E. coli*, I elucidated the correlation between the nitrite concentration and the number of *E. coli*, thereby building the relationship between nitrite concentration and the possibility of a UTI. I also wanted to use this method to simultaneously detect the LE and LF to form a detecting array; however, given my limited time, this aim was not accomplished.

3.1 Sampling module

Developing and necessories for sampling module was done, finally, extraction from gel-based diaper seems promising. Diaper was investigated by me including the layers, absorption and methods to extract the urine. Experiments on the absoption ability and methods of injecting salt solution to release the urine from diaper were done by me in the bioMEMS lab.

3.1.1 Introduction

Obtaining samples from older persons may be difficult who are unable to control voiding or cooperate, in-and-out catheterization and suprapubic aspiration have

therefore become standard methods for urine collection. These techniques can, however, cause pain or discomfort and induce a small risk of infection. Moreover, they are also difficult to perform and more time- and resource -consuming. To overcome these disadvantages there is a need for alternative urine collection methods in older adults.

In men, the use of condom catheters to obtain a urine specimen for culture is most promising. As an alternative to the external catheter in men, external urine collection devices have been developed for urine sampling in adult women. Urine culture collection from disposable nappies has been used for urine collection in children. This technique has now been applied to geriatric care where it can be used with incontinence pads and adult diapers. Urine sampling from disposable diapers can be extracted by pressing over a sterile flask using diapers without an ultra-absorbent gel. Based in some studies, this urine sampling technique is a fairly reliable method for use in severely incontinent elderly women.

In 1991, Ahmad *et al.* [55] collected urine from disposable diapers by compression of wet nappy fibers within a 20ml syringe. In this study, diapers containing gel beads were avoided. The results showed a reduced number of red and white cells in urine collected from the diaper, but unchanged bacterial counting. Collection of urine samples was performed less than 4 hours after diaper wetting. This extraction method proved to be an inexpensive, rapid and simple method of urine collection. Urine extraction from the diaper lining layer was tom away and the damp fibers pushed into the barrel of a standard 20 ml disposable syringe; Urine was obtained by replacement of the plunger and compression of the fibers.

However this cannot be generalized to other types of diapers, such as gel-based ones, which is currently taking up most of the market.[56]. In a non-clinical study, researchers poured urine samples over gel-based diapers, bisected a sample of the diaper's material and compared microbiological results with those from the original urine specimens. A good sensitivity (100%) and specificity (97%) was found. Extraction from gel-based diapers also sounds promising, but needs further exploration.[56]

To assess different aspects of the technique, several experiments were performed. Two popular brands of diapers that contain polyacrylate polymers were tested. Georgia-pacific Nordic As, size 8 disposable diapers (Oslo, Norway) and METSA TISSUE AS, size 4 disposable diapers (Oslo, Norway) were purchased from a local grocer and tested, both of which contain polymer gels.

3.1.2 Layers investigation

A typical commercially available disposable diaper contains several parts: an absorbent pad made of polyacrylate absorbent powder mixed in fluffy wood cellulose fibers, a porous polypropylene top and back sheet, two lateral tapes, a frontal tape, and elastic around the leg area. As people become older, the immune systems are more easily compromised. It is important to keep those who use diapers as clean and dry as possible. There is also a chance of loss of skin integrity, which can lead to skin

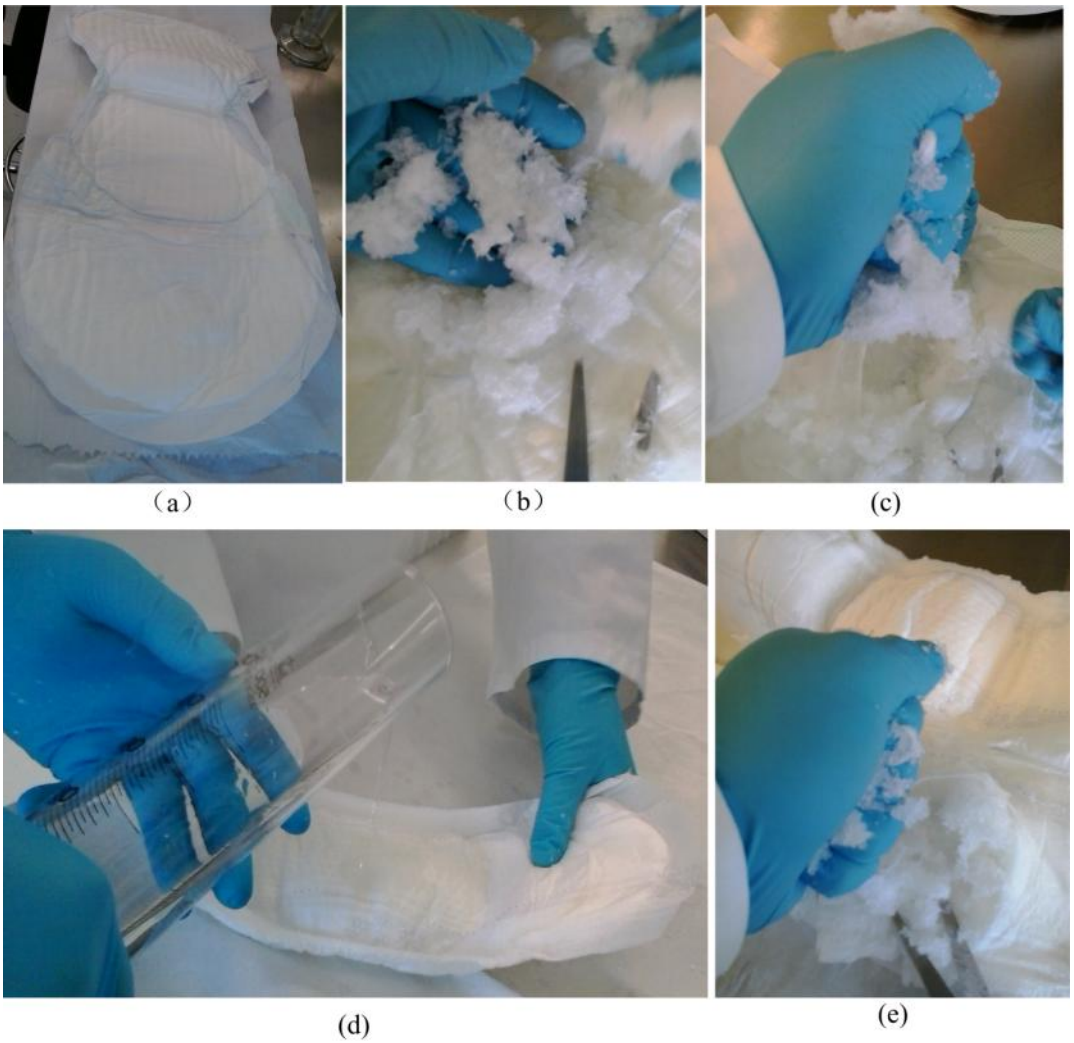


Figure 3.0-1 Absorption ability of commercial diaper. (a) Big diaper. (b) Gel beads soaked with water. (c) Failed attempt to squeeze water from the gel beads in the big diaper. (d) Small diaper being soaked with water. (e) Failed attempt to squeeze water from the gel beads in the small diaper.

breakdown whenever a soiled or wet diaper is not changed frequently, which in turn leads to infections via the skin. The diaper should have an absorbent wicking layer because this will help keep moisture away from the skin longer. The prolonged use of diapers is not as great of a factor as is the lack of regular/frequent changing.

3.1.3 Absorption ability of commercial diaper

Two different sized gel -based diapers were wetted with 500 ml of water, corresponding to the normal voided volume by a healthy adult, and then the inner side of the diaper was bisected along the long axis with sterile scissors. Both diapers showed high absorption ability. However, it was impossible to physically extract liquid out of the gel beads in diapers by squeezing the gel either with hands alone or with a syringe. To obtain the urine sample from the diaper, the sampling modules should be carefully designed, and diapers with internal polymer gel beads should be avoided. The details is as Figure 3.1 shows.

3.1.4 Maximum volume of urine absorbed by the diaper

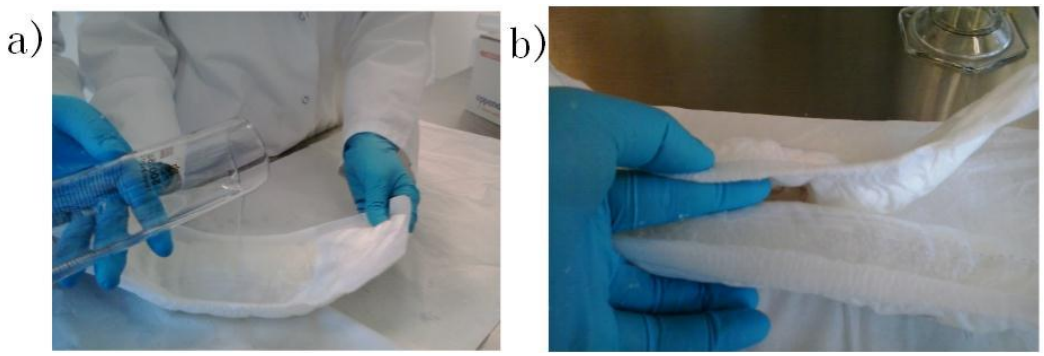


Figure 3.0-3 Ability of a diaper to absorb urine. a) Water was poured onto a small diaper. b) Compare the diapers before and after absorbing the maximum amount of water.

In this experiment, METSA TISSUE AS size 4 disposable diapers (Oslo, Norway) were used, and water was poured on the middle area of the diaper (Figure 3.2 a). The water was totally absorbed, and the shape is presented in Figure 3.2 b. At this moment, 825 ml of water was absorbed; no more water could be added. The normal volume of urine expelled by our volunteers was approximately 150-250 ml, so the diaper can completely absorb the urine without leaking.

3.1.5 Principle for the gel strong absorption

The Principal of this Amazing water absorber inside diaper which holds 200-300 times its mass in water. The reaction equation are as below (equation 2) :

$$C_2H_3COOH + NaOH \rightarrow C_2H_3COONa + H_2O \quad (2)$$

C_2H_3COONa (Sodium acrylate) then is polymerized into long chains of sodium polyacrylate ($[CH_2–CH–(COONa)–]n$).

When in powdered state, the polymer chains are coiled; When hydrated, the sodium ion detaches so that the carboxyl groups become negatively charged and repel one another to uncoil the polymer chain to allow more water to associate with more carboxyl groups or sodium atoms; As the polymer continues to uncoil and absorb water, it swells into a gel-like material. As the polymer also has weak cross-link properties, it effectively forms a three dimensional Structure.

3.1.6 Solutions to release

The absorption of the polyacrylate polymers inside one diaper had been proved, which could absorb 825ml water without one droplet leaking out. The wetted gel was put out and done the analyze. Based on one principal of putting Calcium salt solution

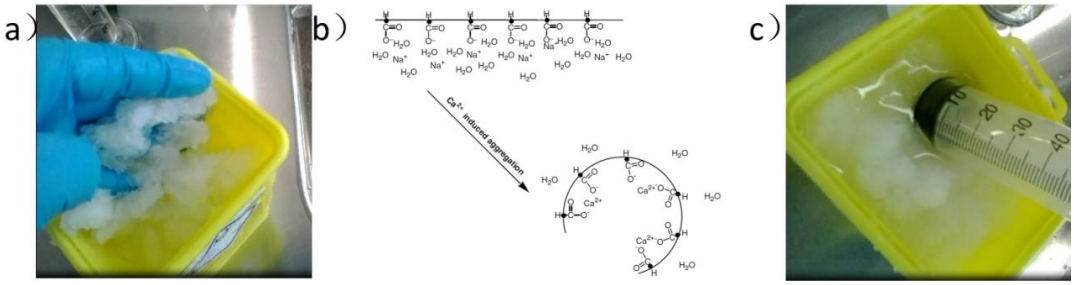


Figure 3.0-4 Urine Extraction from disposable diaper. a) shape of polyacrylate absorbent Powder of one whole diaper after absorbing 500ml DI water; b) principle of the absorption for polyacrylate absorbent Powder(The Ca2+ ions can bind the carboxylate groups of the polymer and neutralizes the polyanions. Due to the hydrophobic nature of the backbone, the polyacrylate chain collapses.)[57] c) water release out after adding 100ml 150 g/l of calcium chloride solution.

can make the poluacrylate chain collapse and leak out the urine [57], the Ca2+ ions bind the carboxylate groups of the polymer and neutralizes the polyanions, shown as Figure 3.3 b). Due to the hydrophobic nature of the backbone, the polyacrylate chain collapses. Combined static and dynamic light scattering suggests a compact spherical shape of the collapsed polymer.

100ml 150 g/l of calcium chloride solution was added and finally get 480ml salt solution by Syringe, as Figure 3.3 c)which provides the solution for the next steps.

3.2 Cell culture

E.coli bacteria were cultured, and finally artificial urine was made by mixing different numbers of E.coli and the fresh urine.

Solid and liquid mediums were made using the materials below: LB AGAR，MILLER Large Granules (Components (per liter): Tryptone 10g, Sodium chloride 10g, Agar 12g); LB Broth, Miller Molecular Genetics Granular; 20n of 250ml Erlenmeyer flasks for liquid culture; 2 of 100ml measuring cylinders; 2 bags of Petri dishes for solid culture; Aluminum foil; 20 of Cork with holes; Marking tape; Blue stopper flasks. Figure 3.4 shows the process for preparation of the solid and liquid mediums

Culture medium were also made in the lab. As for the solid medium, we weigh the nutrition with the Agar (18.5125g-0.215g), diluted into the distilled water of 498ml.; to mixture, shake with hands or the shake with magnetic stirrer for 1min.

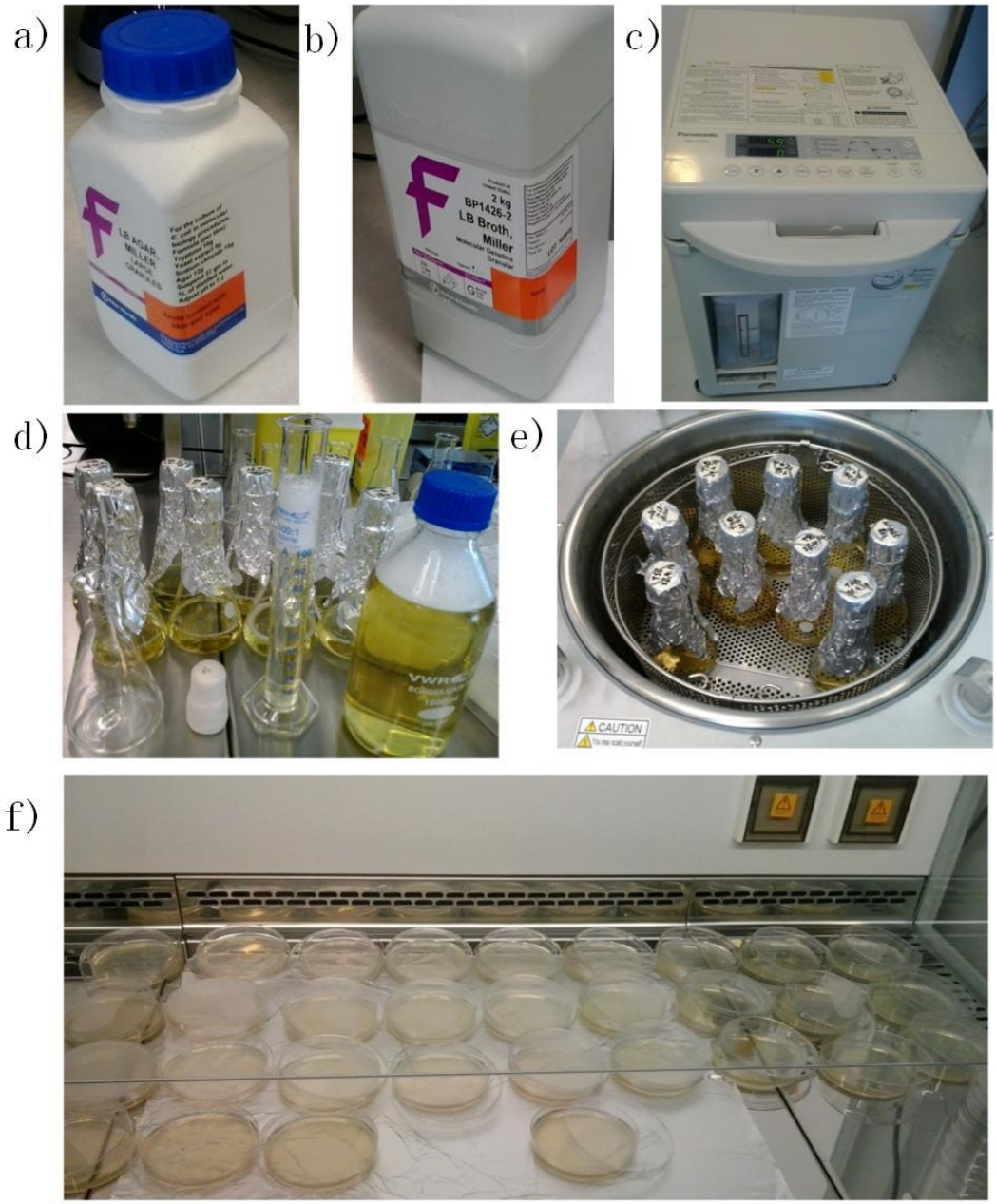


Figure 3.0-5 Solid and liquid mediums making. a) LB Agar; b) LB Broth; c) autoclave machine sterilize; d)liquid medium; e) treatment of liquid medium using autoclave machine sterilize; f) solid medium

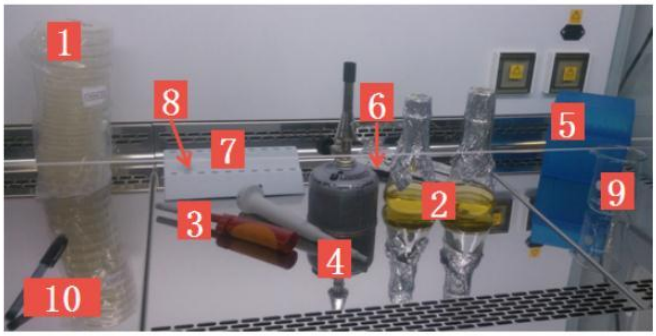
As for the Liquid medium, weigh the nutrition without the Agar (25.0123g-0.0871g, 25.236g-0.1841g), dilute into the distilled water of 1000ml respectively, shake with hands or the shake with magnetic stirrer for 1min; Put the 2L nutrition solution into 20 250ml Erlenmeyer flasks with 100ml volume each, after measuring with the cylinder; Cover the Erlenmeyer flasks with the cork and with the aluminum foil (don't forget to label); Put together with the nutrition with the Agar into the autoclave machine sterilize as Figure 3.4 c) (the autoclave will heat at 125℃at 20min and after 25 minutes keep warm at 55℃).

For the solid cell culture: Write name, date, volume of E.coli and type of culture in the Petri dishes with the agar solid media; Calibrate the pipet for the volume of

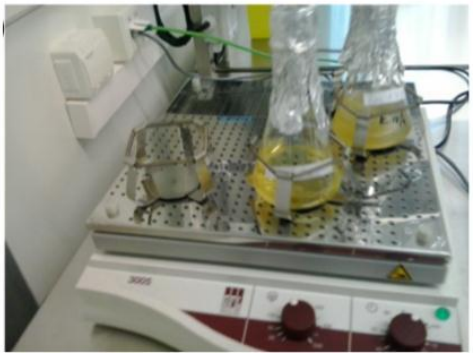
E.coli needed: 50 µL, 100µL, 200 µL; P ut pipet tip in the pipet; Remove the lid of the Petri dishes; Put the E.coli in the pipet; Dispense the E.coli in the solid media; Dispense the pipet tip in the goblet; Spread the sample evenly over the surface of the agar, using a sterile glass spreader; Cover the Petri dish; Isolate with parafilm; Wait 24h for colonies to grow.

a)

1. agar media;
2. liquid media;
3. lighter;
4. pipet;
5. pipet tips;
6. sterile glass spreader;
7. holder;
8. *E.coli*;
9. goblet;
10. parafilm;
11. pen.



b)



c)

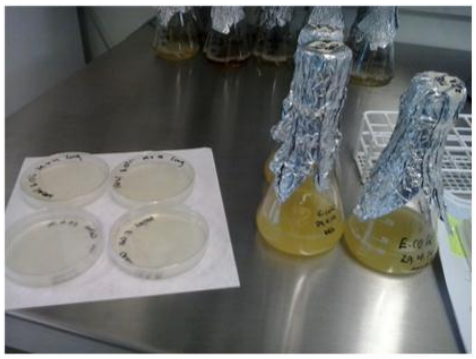


Figure 3.0-6 Solid and liquid cell culture. a) items needed for the cell culture; b) grow of the cell in the medium; c) the cultured cells both in the solid and liquid medium

For the liquid cell culture: Write name, date, volume of E.coli and type of culture in the Erlenmeyer flask with the liquid media; Calibrate the pipet for 100µL; Put pipet tip in the pipet; Open the liquid cell culture, burning the opening; Put the E.coli in the pipet; Dispense the E.coli in the liquid media; Dispense the pipet tip in the goblet; Burn the opening of the Erlenmeyer flask that contains the liquid media and the E.coli, as well as the aluminum foil and cork; cover the Erlenmeyer flask with the cork and the aluminum foil; Put in the mixer at 150rpm for 24h.

There are 3 counting methods, one is using the microscopy total cell count as Figure 3.6 a), another is to using the viable counting as Figure 3.6 b and 3.6 c. Firstly, the cultured media with the unknown number was dilute to 10, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and then they were put into the plate with solid medium distributed. After growth for over 12 h, the cell CFU would be visible, the number time its corresponding dilution time would be the approximate number, after preparation every result from every group, the number can be counted out.

The third method is turbidity which a standard Curve should be generated before the measuring.

E. coli was cultured and harvested at a constant concentration of 3.2×10^{8}, and then the *E. coli* sample was mixed with 150 ml of human urine from a health volunteer at 37℃.

3.3 Detection of Urinary Tract Infections using a lab-on-chip device by measuring the photons emitted from ATP bioluminescence

Based on one chip designed which is a continuous sensing of acute toxicants in Drinking Water [58], a updated design of silicon chip composed of four groups of Y shape internal ribbed micro mixer, T-conjunction droplet generator, TD-Cs and chambers was done. Several investigations and COMSOL simulations had been done on the T-junction droplet generator, Y shape internal ribbed micro mixer and measurement of ATP. The four micro mixers recreate optimal mixture conditions for the sample, sensor cells and buffer solution in the top side while the T-junction droplet generator encapsulates them inside aqueous droplets separated by air. The droplet flow was investigated through numerical simulations since it represents an innovative two-phase flow that allows to oxygenate living cells and to operate at lower pressure than the common two-phase flow [59].The mixed solutions are drove by the O_2 form the central utilizing SFA to the different TD-Cs which provides the time-consuming platforms for Fluorescence. The concentration of toxicants in water can be measured through a small observation chamber and a penetrated chamber with fixed volumes, and then light intensity of the observation chamber is measured on time. The photomultiplier tube (PMT) based detection module will be also installed for quantify weak signals of the bioluminescence reaction (quorum sensing) [60].

With the help of the mentioned lab-on-chip, several experiments were done to prove the feasibility to detect the UTI. Commercial ATP assay was used to test the chip; After building the whole system with the PMT detector and Labview™, the system was also tested by the ATP standard solution. Finally, artificial urine was made and after reading the intensity of photons emitted from the E.coli cells by PMT, the level of the bacteria which stands for the possibility of UTI can be reflected by the value. The method was shown to be feasible for the UTI detection. However, since its high fabrication process and the ATP biomarker is not sensitive enough for the realiable UTI possibility determination, further work should be done to improve the lab-on-chip method.

3.3.1 Introduction

Photomultiplier tube (PMT) based detection module is also installed for quantify weak signals of the bioluminescence reaction (quorum sensing) [60]. It has been instantaneously recording the maximum bioluminescent light signal emitted from the whole reaction process by measuring the changes of the voltages on the Labview™. Usage of PMT by measuring wavelength to count luminance photons one by one had been studied and would be used in the future researches, which has better sensitivity and seems promising. Considering the structure of one chip which is quite suitable for the firefly luciferin-luciferase method and PMT detection, I applied this chip for the application of detecting UTI. The existed chip is composed of two counter-flow micromixers, a T-junction droplet generator and time delay channels (TD-Cs). Urine sample which had been pretreated and nucleotide releasing buffer were imported into the first micro mixer at rate of 1μL/min where the ATP would be totally released. And then the mixed solution and *Luciferin* were imported into the second micromixer at the rate of 1.8 μL/ min, after which the droplet generator encapsulated them inside aqueous droplets separated by air. Air flow was the disperse medium, which could guarantee sufficient oxygen supply for the cells in droplets and also control the time that solution stayed in the channel of TD-Cs. The TD-Cs is a round spiral channel which can provide the platform for the detection of the PMT. The system showed high reliability and stability through numerical and experimental investigations. In the microfluidic domain, the analyzer is based on continuous flow, using syringe pumps. The system includes the flow in the chip, the PMT photon detecting and electrical circuit to connect to the PC, and the software including Labview™ to visualize the data. Continuously, it had been tested by the ATP standard solution. After which, fresh normal Urine samples had been mixed with different amounts of *E. Coli* cells and tested, which proved feasibility of detecting UTI.

This part have successfully given out the concept of using microfluidic chip as a platform providing bioluminescent reaction happening and using the PMT system detecting the luminance photons emitted and finally getting the signals for detecting UTI accurately and quickly.

3.3.1 Experimental setup

ATP Assay kit was used to prepare the ATP standard solution. One novel chip was drawn by me using the solidworks, it was designed by me and the mixer efficient and SFA transporting are also simulated by me. The whole system was installed with the existed chip and PMT detector.

3.3.2.1 Preparation of biological samples

ATP Assay Kit (119107-1KIT) was purchased from Merck Chemicals Ltd. (Padge Road, Beeston, N G9 2JR Nottingham, United Kingdom). The reagent is constituted of one vial of Luciferase ATP Monitoring Enzyme, Enzyme Reconstitution Buffer, a bottle of Nucleotide Releasing Reagent, one vial of ATP Standard, and a user protocol. The assay is based on the firefly luciferase-catalyzed oxidation of D-luciferin in the presence of ATP and oxygen, where the amount of ATP is quantified by the amount of light produced.

Prepare the ATP standard solution by dissolving 1mg ATP into 1ml of H_2O, and store it in the fridge at -20℃, which will be used to detect the whole system. Remove different volumes of normal urine medium and 10^4-10^6 *E.coli* cells cultured in Luria Broth (LB) medium with 10g/L Tryptone, 5g/L Yeast extract and 10g/L NaCl as inlet 1, nucleotide releasing buffer as inlet 2,they are mixed at first Micromixer for about 5min at room temperature. Reconstitute ATP Monitoring Enzyme with 2000μL of the Enzyme Reconstitution Buffer freeze at -70℃for future use and then use 1ul ATP monitoring enzyme being diluted with 49ul Nucleodtide releasing buffer as inlet 3 for the chip. ATP and enzyme solutions were pre-treated by Multifunctional sample preparation kit and injected into the microfluidic chip at different flow rates through 3 inlet ports separately.

3.3.2.2 Chip design and configuration

This is a novel silicon chip with two sides which mainly has four groups of Y shape internal ribbed micro mixer, T-junction droplet generator, TD-C and chambers.

For the top side, as Figure 3.6 a), internal ribbed micro mixers which inherit the design of micro-

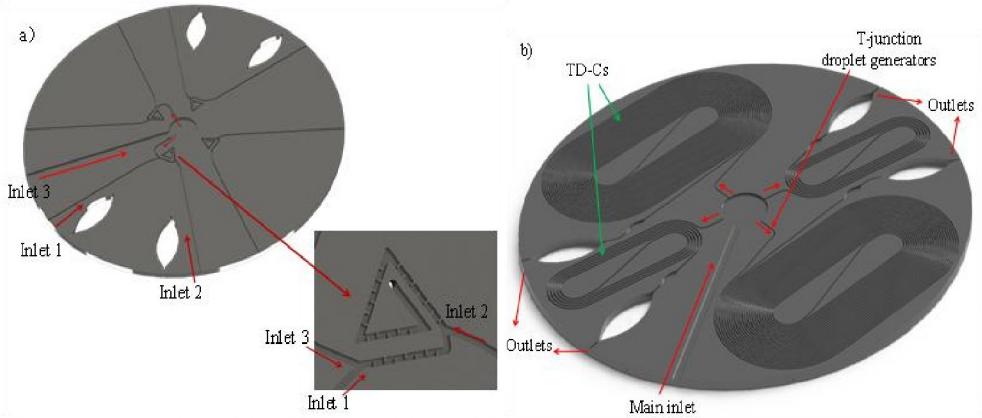


Figure 3.6 Novel Double-sided multichannel living-cell-based Microfluidics chip; (a) top side with the structure of the mixer; (b) bottom side.

concentrator to force two fluids to flow in the same internal ribbed channel, improving the mixing efficiency. The micro mixers designed has successfully made three solutions mixed. For the bottom side, as Figure 3.6 b), O_2 will go through the

main inlet firstly to the center and disperse into 4 channels at the same velocity. With the help of T-junction droplet generator and SFA, the encapsulated droplet will be made and brought from the bottom side to the top side, after which it will be broken up and transported to the different TD-Cs at control time and velocity

3.3.2.3 T-conjuction droplet generator

Figure 3.7 shows schematically the geometry of the T-junction. The continuous mixed

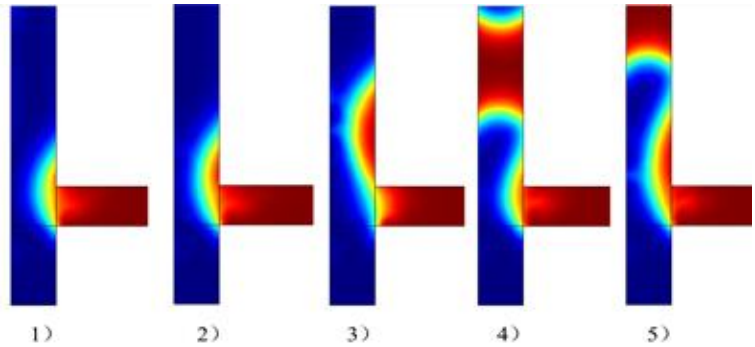


Figure 3.7. Process to generate the droplet.

solutions pass through the channel on the top side of the chip and after

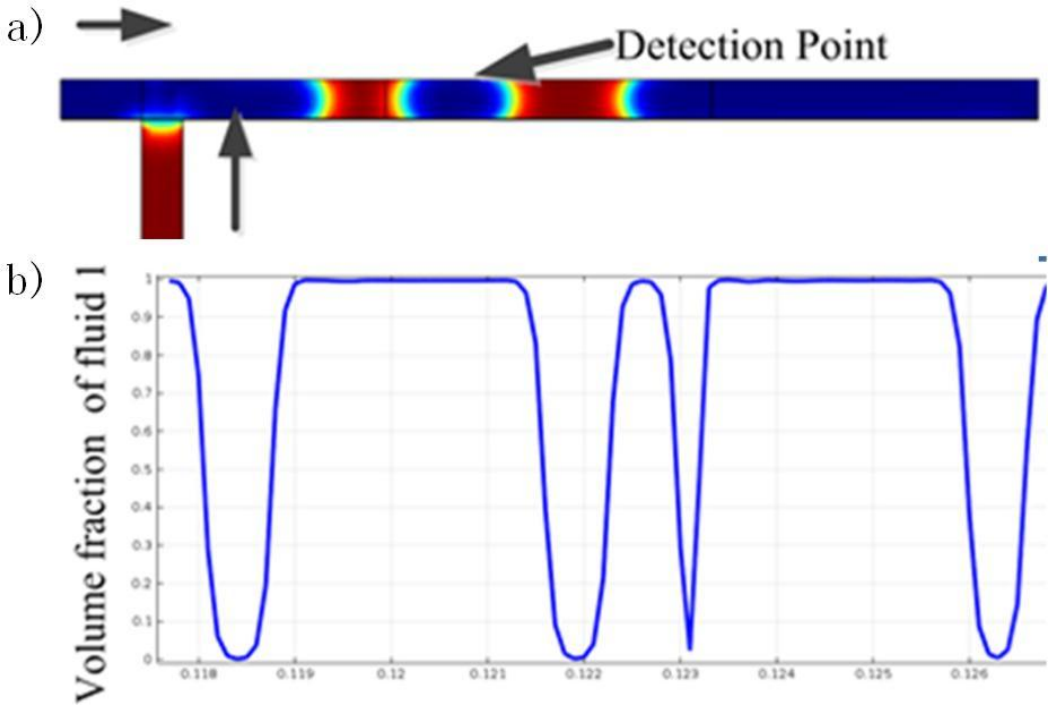


Figure 3.8. Investigation of the T conjunction droplet generation; (a) structure of the conjunction generation; (b) volume fraction of fluid 1 changing with time.

the mixed fluids form an interface at the T junction of the penetrated hole where gas was put in from bottom side. The discontinuous phase stream penetrates into the main channel and a droplet begins to grow; the gas from the center made by SFA in the main channel distorts the droplet into the downstream direction.

Resulting in the interface on the upstream side of the droplet moves downstream and the droplet break appears at the neck connecting the punched hole and main channel. The disconnected liquid droplets flow downstream in the main channel, while the tip of the stream of the discontinuous phase retracts to the end of the T conjunction and the process repeats.

Finally, this process generates uniformly sized droplets (Figure 3.8) and the volume of these droplets can be adjusted by changing the rates of flow.

We are interested in how the sizes of the droplets formed in a T-junction depend on the rates of flow, the viscosity of the continuous and discontinuous fluid (not studied here), and if these droplets can be continuous. So the detection point (0.025, 0.4) has been selected, relations between volume fraction and fluid with time has been drawn.

3.3.2.4 Y shape internal ribbed micromixers

Different mixers had been investigated for a long time, the Y shape internal ribbed micro mixer which have been proved its mixture efficiency had been used by me.

To make the three Reagents from the middle and two edges possible to be mixed, two Y shape mixers were used; to make the mixing totally in a small space and convenient to move them to another side, the structure in the Figure 3.9 a) was designed. And simple COMSOL simulation of shear force distribution had been done to check its killing capacity to the cells, as Figure 3.9b)

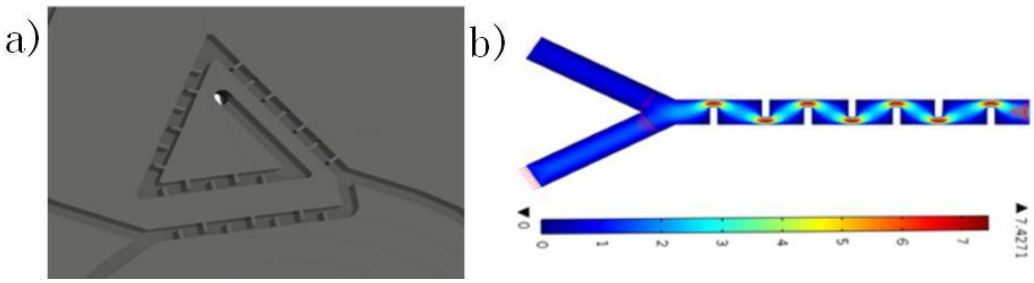


Figure 3.9 Two-dimensional mixing properties of the counter-flow micro mixer; (a) structure of the mixer; (b) shear rate distribution around a single counter-flow unit.

3.3.2.5 Installation of the microfluidic chip

The microfludic chip was installed in the lab. Sygen pumbs were used for providng the force; samples and O2 were injected as 3 inlets with the help of sygen pumbs to go in as Figure 3.10 a) shows. Flow of water was used to check the well- installation of the microfludic chip.

In this study, a designed chip as shown in Fig. 3.10 b), composed of two micromixers, a T-junction droplet generator and six TD-Cs was used. The two micromixers recreate optimal environmental conditions for the release of the ATP

from constant cells while the droplet generator encapsulates them inside aqueous droplets separated by air. The droplet flow had been investigated through numerical simulation since it represents an innovative two-phase flow that allows to oxygenate living cells and to operate at lower pressure than the common two-phase flow. The concentration of ATP in different levels of E. coli for different urine samples can be measured by quantifying the light intensity at the TD-Cs.

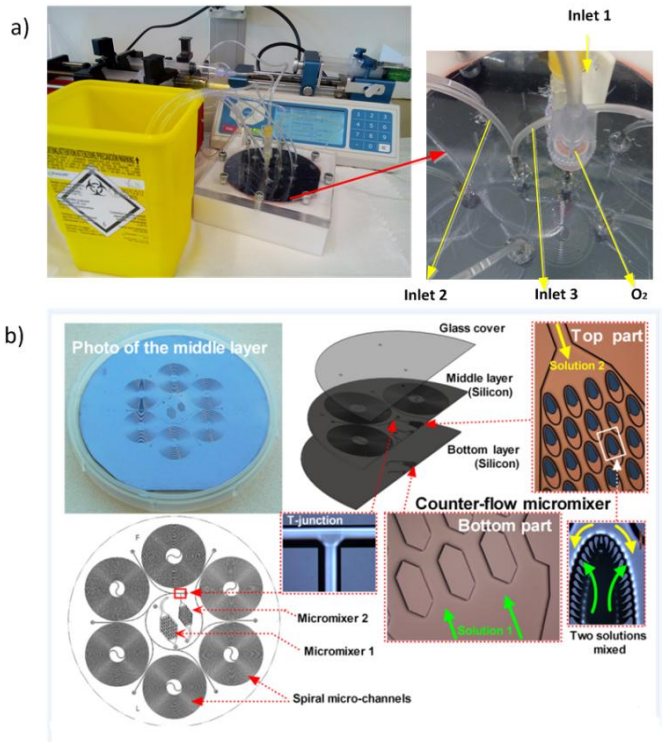


Figure 3. 10 Description of the cell-based LOC device; (a).Application of LOC chip; inlet 1 for the samples, inlet 2 for the nucleotide releasing buffer and inlet 3 for the mixed ATP monitoring enzyme; (b) With permission from Dr. Xinyan Zhao.Construction of the cell-based LOC device [18]; The chip has three layers, but the three major structures are in the middle layer, including two counter-flow micromixers, a T-junction droplet generator and six spiral micro-channels. A photo of the device is shown in the top left corner. The schematic and micrographs of the counter-flow micromixer are illustrated at the top right part, which houses the counter-flow units (on the middle layer) and the inlet channels (on the bottom layer). The tiny inlet port is located in the center of the counter-flow unit. The sample, buffer solution and the cell suspension are mixed and used to form droplet within the air flow.

3.3.2.6 Building of the detecting system

The whole system is shown in Figure 3.11, which includes the microfluidic chip, the PMT photon detector and read-out circuit connecting to a PC with the software of Labview to visualize the data. Form the voltage appearing on the Labview which reflects the

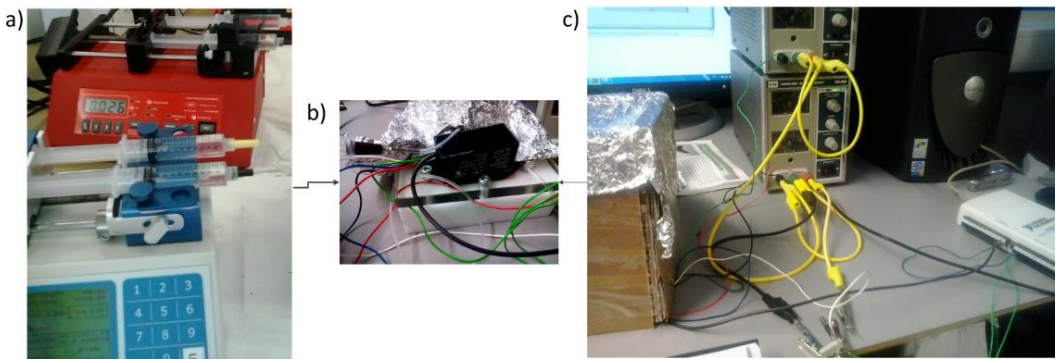


Figure 3. 11 Testing of the whole system; (a).the flow of the chip. (b).the PMT photon detecting; (c). electrical circuit to connect to the PC and the software including Labview™ to visualize the data.

intensity of the light, getting the relationship of the voltage with the concentration of the ATP. With the decrease of the concentration of the ATP that applied, we can detect the lowest detection concentration. After the whole system being built, different concentration of *E.coli* in the urine sample was transported to the chip system; after going through the chip, the signal was recorded with the PMT detecting system. After that, the signals were visualized by Labview on the PC. the data of UTI can easily observed and shared.

3.3.2.7 Bioluminescent Detection tests of ATP

ATP and enzyme solutions were pre-treated by Multifunctional sample preparation kit and injected into the microfluidic chip at different flow rates through 3 inlet ports separately (Enzyme goes through inlet 3 into mixer, ATP goes at inlet1 and acid buffer solution goes at inlet 2). In optimized conditions the method will be successfully applied to measure the concentration of ATP by evaluating through the intensity of emitted light measured by the PMT-based detection module. After the observation chamber is filled with gas-liquid two-phase flow, the detection module starts to work. The luminescence data will be calculated using Microsoft Excel® and OriginPro® software.

3.3.2 RESULTS AND DISCUSSION

3.3.2.1 SFA used with the T conjunction droplet generator

T conjunction droplet generator is useful for not only encapsulating cell sensors to the droplets but also making a bridge to the bottom side to the top side. SFA plays a vital role for making droplets generated by the T conjunction moving periodically and continuously, which can make the system stable. It can be seen form the COMSOL simulation that the volume of liquid droplets and gas are almost periodic, therefore, the system is stable.

3.3.2.2 Y shape internal ribbed micromixers

The mixer had been designed as picture Fig. 5a shown, which was selected for reaching a biggest mixture effects comparing with some other mixers, so mixture simulation has been neglected. COMSOL simulation also had been done for the shear rate change when reagents meet with the rigs and the maximum shear force magnitude will not be harmful to the cells. The design is successful for it modifies one mixer made before which has proven its mixture abilities and makes the possibility that mix reagents totally in the small space, finally making the droplet generator happening with the T junction droplet generator.

3.3.3.3 Testing the mixture efficiency of the microchip

Several experiments had been done for the microfluidic features of the chip including the refining microfiltration and different inlets rates determine the mixture effect. Finally when urine sample form the inlet1 being at rate of 1μl/min, nucleotide releasing buffer solution flowing at 1μL/min from inlet 2, and mixed with mixed ATP monitoring enzyme flowing at 1.8μL/min from inlet 3 and mixed solution was capsuled by the Air flow at the rate of 1.5μL/min, which provided a stable flow and meeting with the optimal condition for chemical reaction and detection.

3.3.3.4 Testing of the whole system

Software of this system was designed, by measuring the output which is linearly corresponding with signal intensity. Hardware of this system was also designed, using two power supplies, one constant 4.7kΩ resistor, one 1kΩ potential resistor, several wires to connect and DAQ National Instruments. Both the testing of the software and hardware of the PMT and basic measurement of the voltage reflecting the

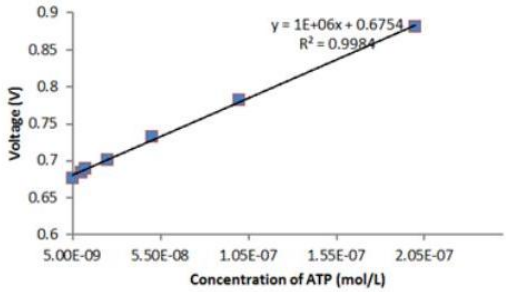


Figure 3.0-7 ATP standard solution was used to test of the whole system.

bioluminance light intensity emitted from the ATP standard solution had been done, which proved its application.

When the ratio of ATP to enzyme solution was fixed, the optimum flow rates of enzyme, ATP, and enzyme solution was 3.6, 8.2, and 3.6μl/min, respectively. The optimal total flow rate was 15.2 μ l /min.

ATP standard serials of solutions were made into the concentration 10^{-9}、10^{-8}、10^{-7}、10^{-6}、10^{-5}mol/ml respectively. With the help of the PMT detection, the standard curve of ATP concentration and calculated value of the light intensity has been drawn as Figure 3.12.

3.3.3.5 Measurement of UTI

Different numbers of cell cultured E.coli was mixed with fresh urine as Figure 14a).

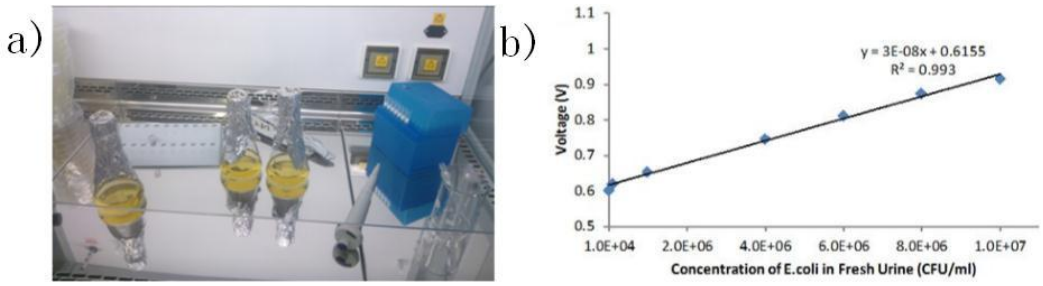


Figure 3.0-8 Detecting of the Urine with different amounts of E.coli

then the artificial urine samples were transport them into the system separately. As Figure 3.13 shown, the voltage has the linear trend with the different numbers of the *E. coli*, which proves feasibility of detecting the UTI. *E.coli* cells were kept for 8 hours in the fridge at 4℃， however no signals were detected, which prove that the system can only detect the fresh samples.

3.3.3.7 The simply and more precise PMT detection system

The aim of this paper is to apply the PMT detecting system to read the photons of the ATP luminance. A lot of investments have been done, however, what the experiments had been down is to have built a system using Labview™ visualized the dates form the luminance accumulated light by measuring the voltage appearing. In the latter work, improvement of PMT detection system will be done, the light signal can be measured by photons one by one, thereby creating the highest sensitivity and other methods will also be tried, including polycarbazole photodetector.

3.3.3.8 The efficiency of the detecting UTI on lab-on-chip

Combing with the ATP assay and the microfluidic chip is designed to detect the UTI, which has the advantages of both fast reliable detecting and more precisely with very lower limit detections. Using LOC also prevents the poor stability and high cost of luciferase and d-luciferin in bioluminescent reactions. And it can also reused, which makes the less expenses. Moreover, because of lab-on-chip which is small and portable, more related experiments could be investigated based on this platform and it may render the complicated immuno-NASBA assays convenient to common users without special training.

3.3.4 Conclusion

ATP luminance method to detect UTI was applied on the microfluidic chip. One ATP commercial assay kit and one designed microchip had been combined and with the help of the PMT detecting system, the UTI could be quickly and precisely detected by reading the visualized data from the luminance photons emitted from ATP firefly luminance reaction. This combined method is promising because microchip can protect the ATP away from polluting to get an accurate result, moreover, by reading photons, the lower concentration can be figured out without urine culture, which provides the quick detection and saves high cost of *luciferase* and *d-luciferin* in bioluminescent experiments. In this paper, only simple experiments have been done for this promising conception, the more precise experiments will be done in the future.

The work focused on the design of one online lab-on-chip continuously monitoring platform system which can be helpful for medical, pharmaceutical and environmental monitoring. And simple Bioluminescent Measurements of ATP had firstly been used which proved to be successful. T conjunction operator combing with the SFA makes the whole system continuous and Y shape internal ribbed micro mixers were used to reach a good mixture effects in a small space. Basic simulations and the measurement had been done, which proved it can be the successful monitoring useful tool.

With the development of luminescent techniques applied to bio analysis, it is important to find a simple and reliable method to do the measurements. PMT has been used in this paper; some other methods will also be tried, including polycarbazole photodetector and liquid scintillation counter. And to meet with the aim of multifunctional design, more couples of important toxicants and biosensors will be texted in my further work, especially on the UTIs. I believe this design is the successful connecting between medicine fields to the physical analyzers.

3.4 Diaper-based UTI test for elderly people via electrochemical sensor measuring of $NO2^-$

An existing nitrite electrochemical sensor was available in the lab; I investigated various electrochemical sensors and how they work. Then, the electrochemical nitrite sensor was assembled and calibrated, and the artificial urine sample was detected; the feasibility of the electrochemical nitrite sensor, including the errors effect, was checked and was approximately -5.1~2.3%; the ability to detect an artificial UTI urine sample is approximately 95.5%; the approximate relationship between the number of E. coli and the electrode potential was built as E=228.3193-3.78225×Ln (N+2.29101e6), which represented the relationship between the possibility of a UTI and the measurement. Finally, the electrochemical sensor array was conceptualized

and designed to enable the measurement of different biomarkers to maximize the ability to detect a UTI and to present the data regarding the possibility of a UTI directly on the screen. Furthermore, this biosensor was developed to be easily used and transported for hospitalized patients and those at home.

3.4.1 Introduction

Nowadays creating a new equipment to detect the nitrite ions (NO2-) , which is made almost only by *E. coli* and appears in UTI urine quickly and precisely seems quite promising.

Generally, membrane-based biosensor can be separated into two parts: Potentiometric and Voltammetry methods. In this paper, Ion selective sensor which belongs to potentiometric had been used which has three main basic components: a) reference electrode: gives reference for potential measurement; b) indicator electrode: where species of interest is measured; c) potential measuring device, as Fig. 1.a) shows and it is based on measurements of the potential of electrochemical cells in the absence of appreciable currents as Fig. 1.b) shows. In the application as in as in Nernst equation [61]

$$E = E_0 + (2.303RT/nF) \times \text{Log}(a) \quad (3)$$

Where E = the total potential (in mV) developed between the sensing and reference electrodes. E_0 is a constant which is characteristic of the particular ISE/reference pair. (It is the sum of all the liquid junction potentials in the electrochemical cell); 2.303 is the conversion factor from natural to base10 logarithm;R is the Gas Constant (8.314 joules/degree/mole); T is the Absolute Temperature; n is the charge on the ion (with sign); F is the Faraday Constant (96,500 coulombs per mole); Log(a) is the logarithm of the activity of the measured ion.Different concentration of Nitrite solution with 1000ppm, 100ppm, 10pm, 1pm,

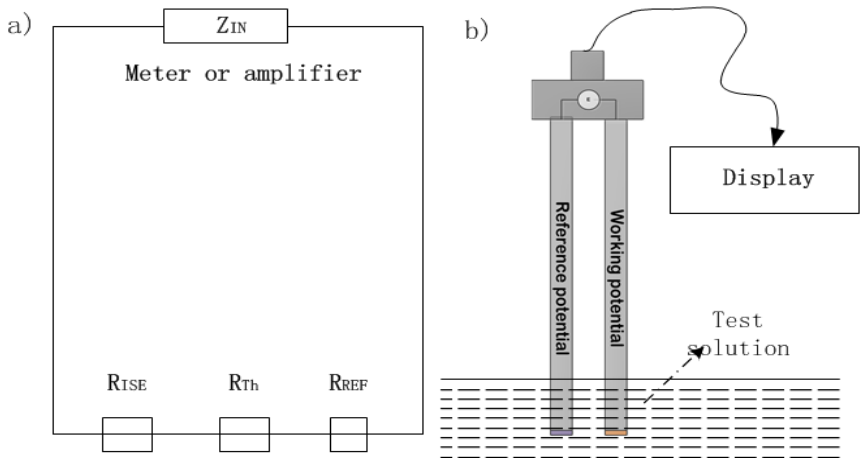


Figure 3.0-9 Principle of electrochemical sensor. a) Three main basic components; b) detect process.

0.1ppm and 0.01ppm were used to calibrate the electrochemical sensor, which proves the Feasibility for its application to detect the different concentration of nitrite.

3.4.2 Assemble and calibrate electrochemical sensor.

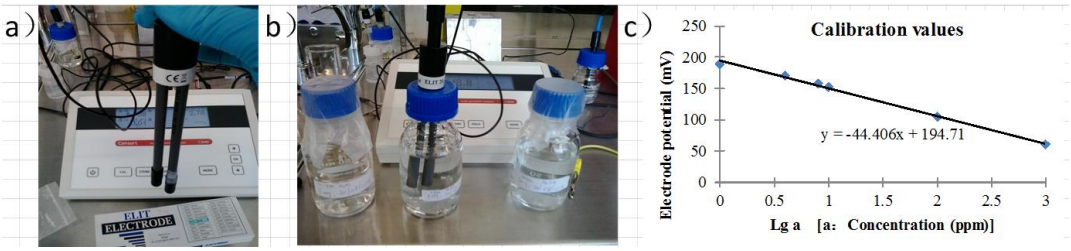


Figure 3.0-10 Assemble and calibrate the electrochemical sensor. a) Assemble the commercial electrochemical sensor; b) using different concentration of nitrite solutions to calibration the electrochemical sensor ;C） results to show the feasibility of the sensor.

3.4.3 Experimental section

3.4.3.1 Detect the UTI.

Fresh urine was mixed with different volume $NaNO2^-$ and prepared. They were detected and measured, the results shows that the electrochemical sensor has the ability to detect the amounts of nitrite concentration in the urine. If there is one database for the nitrite of UTI, there will be a promising marker for detecting UTI precisely.

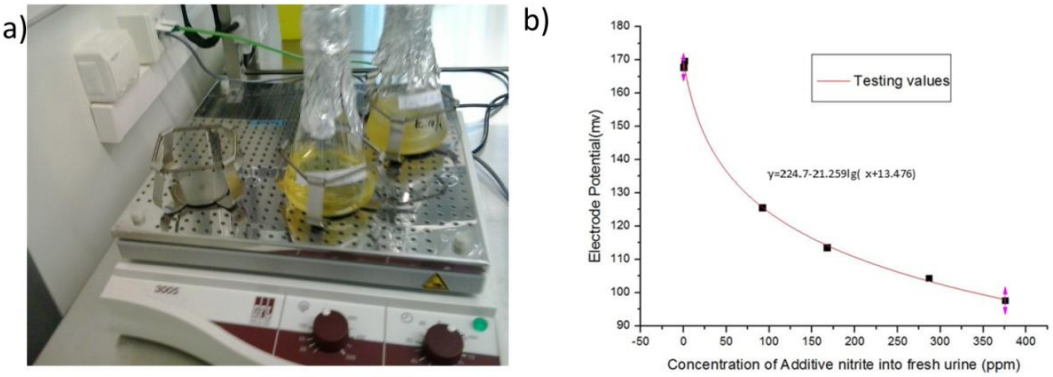


Figure 3.0-11 Tests of Electrochemical sensor. a) Preparation of the urine samples with different constant concentration of nitrite; b) using different concentration of Nitrite solution to mix with the fresh urine to test the electrochemical sensor

There are several factors which will affect the precise of electrochemical sensor's nitrite detection, including noise of the detection machine, temperature of the solution, urine samples from different human beings.

Error of this electrochemical sensor keeps the range at -13.2%~5.1%;Temperature bring error to the detection, which should be constant during the experiments； Different urine sample has the different value. For the health urine samples, ranging from 147.3mv to 183.6mv, with the average value 169.875; for

the artificial UTI urine samples, ranging from 114.9mv to 137.2mv, with the average value 124.51mv. There will still be little chance that there is no nitrate in the urine sample.

We can detect if someone has the UTI by the values above, which shows its feasibility to detect UTI out.

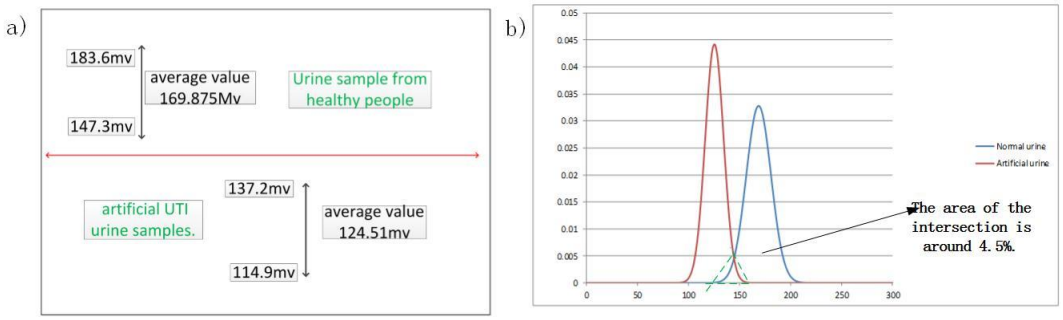


Figure 3.17 Detect the possibilities of electrochemical nitrite sensor for the UTI. a) The values detected from normal urine and UTI urine; b) the possibilities of the intersection between two groups of detection

Temperature brings error to the detection, which should be constant during the experiment. Different urine sample has the different values. Many samples were detected with different controlled conditions, which gets that errors of this electrochemical. Sensor keeps the range at -5.1~2.3%. 20 normal urine samples from h iealthy people and 20 artificial urine samples by adding nitrite had been test to prove the feasibility of the electrochemical sensor. The results are: for the health urine samples, ranging from 147.3mv to 183.6mv, with the average value 169.875mv; for the artificial UTI urine samples, ranging from 114.9mv to 137.2mv, with the average value 124.51mv, as Figure 3.17 a). Standard distribution was drawn based on the detection values as Figure 3.17 b). The intersection was calculated with the value around 4.5% as which means this electrochemical sensor can differentiate the artificial urine samples from the normal persons' samples by the possibilities at 95.5%.

3.4.3.2 Approach for detecting Kinetics mechanism of converting nitrate from normal diet into nitrite by E.coli

Prepare the *E.coli* (ATCC25922) cells for 1.85×10^{9} CFU/ml; dilute E.coli 85×10^{8} CFU/ml, 1.85×10^{7} CFU/ml , 1.85×10^{6} CFU/ml, with enough human urine of a health volunteer at 37℃for $1.1.85\times10^{5}$ CFU/ml and 1.85×10^{4} CFU/ml at the ration 1: 100 (volume of the culture medium to the volume of urine) respectively. Nitrite was measured by the electrochemical sensor after 24h. Different number of E.coli generated different concentration of nitrite. And then relationship between the numbers of E.coli and electrode potential can be found as equation (4).

$$E=228.3193-3.78225\times L\,n\,(N+2.29101e6) \quad (4)$$

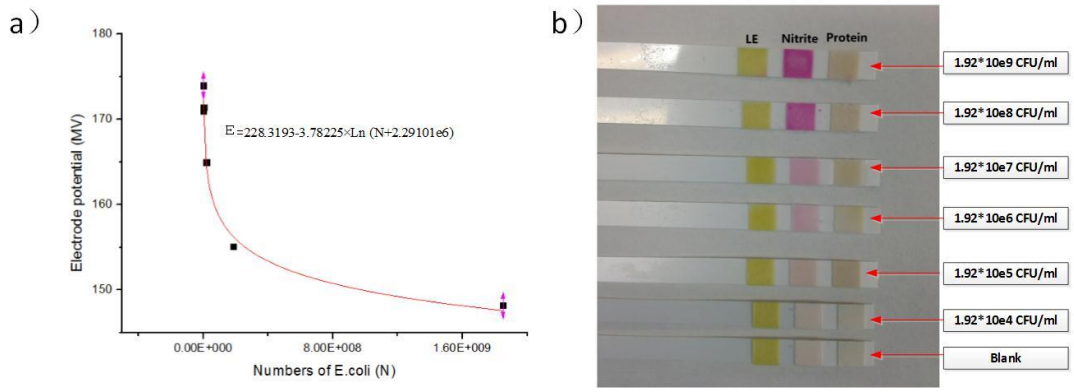


Figure 3.0-12 Approach for detecting Kinetics mechanism of converting nitrate from normal diet into nitrite by *E.coli* a) Relationship between the numbers of *E.coli* and the measured electrode potential; b) verified by the standard normal urine strips.

It has the same equation kind as the Equation (3). Since concentration can be calculated out by the Equation (3) after modifications. Thereby, this test also gave the relationship between the number of *E.coli* and concentration of nitrite in the urine sample, which is almost linear relationship. Finally, the relationship between UTI possibilities and nitrite detection can be made. This proves UTI can be detected quickly and quantitatively by detecting nitrite using electrochemical sensor. Used solutions were tested by standard strips. We found that higher numbers of *E.coli* produce higher concentration of nitrite， as Fig. 3.18 shows.

3.4.4 Concept discussion & future work

3.4.4.1 Nitrite detection for UTI detection

The correlation between the number of E. coli and the nitrite concentration has been investigated, and this correlation can be used to obtain an approximate relationship. However, more specialized experiments should be performed to generate data to build this relationship. Because the number of E. coli in urine corresponds with the nitrite concentration, it was easy for us to use the obtained results to quantitatively diagnose a UTI without cell culture. However, there are still some undetermined issues regarding the detection, including the nitrate content in human food, the time required for E. coli to convert nitrate into nitrite, the urine pH that controls this conversion process, the effect of other bacteria that can generate the nitrite, the temperature and the noise; all of these issues can introduce some level of error into the detection process. However, this type of detection method can be used by home users to determine the possibility of a UTI infection.

3.4.4.2 Electrochemical sensor array for UTI detection

From Figure 18, the feasibility of the sensor to detect UTI out has been proved. From

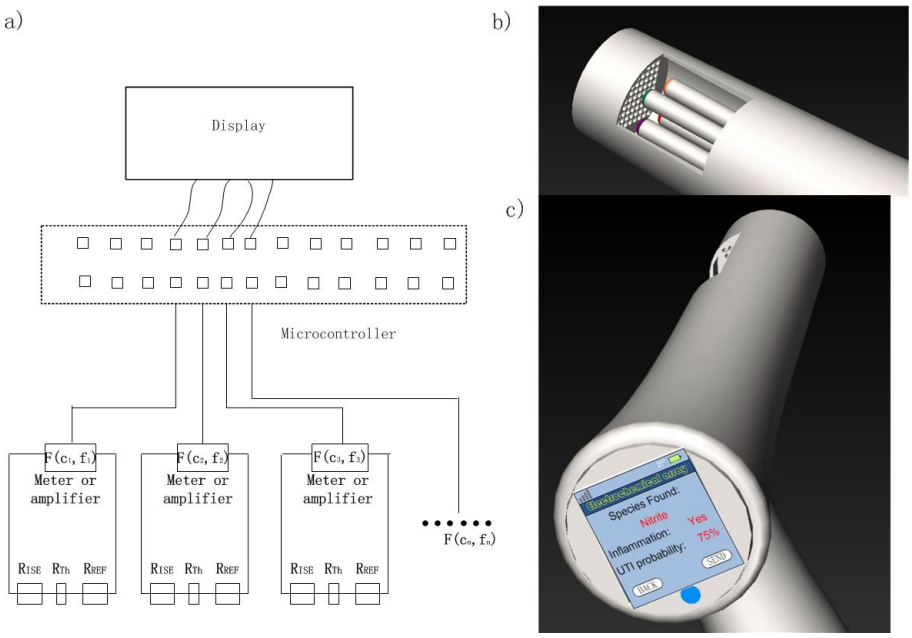


Figure 3.19 Design of electrochemical array. a) diagram of the electrochemical array; b) shape of electrode arrays and the sampling structure; c) the screen of the electrochemical array.

The feasibility of the sensor to detect a UTI was demonstrated (Figure 3). We attempted to build a relationship between nitrite concentration and the number of E. coli (Figure 3.18). Electrochemical sensor arrays can be realized for Rapid Multiplexed Detection of Pathogens. If we use the electrochemical sensor to detect nitrite, we have some ability to detect a UTI. The signal can be frequency dominated, which can be described as F(c, f1). Similarly, we also detect other biomarkers (for example, alcohol, leukocyte enzyme , and urinary lactoferrin) to improve the ability to detect a UTI as a signal function F(c, fx) and report the results on the screen. If we can simultaneously measure the nitrite concentration and the LE or LF quantitatively,

we can measure a UTI with the maximum possibility; this is the same idea as using one paper to detect waterborne pathogens that has been designed and validated to have the maximum possibility . More studies will be conducted to build and fabricate the sensor array, which can analyse several biomarkers and perform integrated automatic sampling (Figure 3.19), rapid detection, and auto-decontamination and to support personalized health information technology (HIT), thus providing a low-cost solution for automatic urinalysis services for both patients in hospitals and home users. Moreover, this sensor can analyse and directly present the data regarding the possibility of a UTI on the screen and can be easily used and transported.

3.4.5 CONCLUSION

In this paper, commercial electrochemical sensor was assembled and calibrated to detect the nitrite in the urine sample. After building the relationship between the nitrite

concentration and the number of *E.coli* bacteria, the electrochemical sensor was used to detect the *E.coli* bacteria, which directly build the relationship between the sensor to the UTI possibilities. Finally, in the discussion part, the conception of electrochemical array was pointed out which can not only detect different biomarkers to make the maximum detection possibilities but also combine with the functions such as automatic sampling, rapid detecting, auto-decontaminating and personalized health information technology (HIT) supporting.

Chapter 4

Fabricating a paper-based lab-on-chip for the point-of-care diagnostics of UTI

This chapter presents a promising alternative method for the microfluidic lab-on-chip point-of-diagnostic detection UTI on diaper. I performed many investigations of the paper, Dipstick, and lateral flow assays and on the microfluidic paper-based analytical lab-on-chip development. I conducted some simple experiments on the normal strips and diaper, which give the basis for their further design and fabrication. Design of this paper-based lab-on-chip was done by Dr. Nuno Pries, Dr. Zhongfang and Master student Chaohao Chen. Material selections were mainly done by Master student Jennifer panugan. However, I pointed out the conception for paper-based lab-on-chip to use for diapers, and different fabrication methods and theoretical studies were also investigated by me. Wax-based fabrication methods to make the hydrophilic channel and hydrophobic barrier were tried by me in the lab. Tests were done for the reaction layer which has the hydrophilic channel and hydrophobic barrier, which proved the design and fabrication for the reaction layer is suitable and successful. Further work will be done on the details of fabricating whole chip and clinical examinations of this product. Moreover, the quantitative readout which Dr. Haakon Karlsen are doing would be invented and data could be transported to the hospitals and persons with most of conveniences.

4.1 Background

To be a competitive device, paper-based assays must meet the following aspects: (i) transport small volumes in a short time, (ii) automatically complete the functions without functional units, (iii) maintain high specialization and detection sensitivity, (iv) be enclosed to avoid contamination of and evaporation from embedded microchannels, and (v) transport the biological samples to the proximal end of the strip where the sample pad is located without losing the components (for example, protein) to keep the nonspecific background low.

Currently, commercial paper material is mainly divided into two categories for POC diagnostics [62]. One is cellulose fibre-based materials, such as filter paper and chromatography paper, which are the key substrates of dipsticks and μPADs [63]. The other is nitrocellulose, which is the main material for lateral flow assays (LFAs) [64]. Paper is rough and hydrophilic; therefore, it is a challenging to utilize paper to quantitatively detect biological samples at the POC. Thus, research on the surface

properties and on surface modifications to reduce the effects on sample transport were of increased importance. The properties of paper that are relevant for microfluidic applications include the surface characteristics, capillary flow rate, pore size, porosity, bed volume and colour. The surface characteristics vary among the chosen materials. Chromatography and filter paper are always chosen due to their low porosity and surface tension. Printing paper has been shown to be an unsuitable material for constructing hydrophilic channels for liquid transport, and kitchen towels have showed that the diffusion in hydrophobized materials is uneven and incomplete [65]. Because the effective concentration of the analyte in the sample is inversely proportional to the square of the change in the flow rate, the capillary flow rate is another key parameter in assay performance [66]. The pore size (nominal or absolute) is related to the size of the particles retained by the filter [67]. The pore size distribution (PSD) defines the range of pore sizes in the membrane and determines the capillary flow rate as a function of the aggregate pore size. In paper-based microfluidics, the bed volume determines the total sample volume that is absorbed by the absorbent pad, so this parameter is especially important. The colour of the paper is important because it plays a role in image analysis. A high-concentration of lignin that consists of chromophores in wood causes paper to discolour and eventually self-destruct [68].

Dipsticks and LFAs are the established assay formats for POC testing. These assays are available with housing units. Nowadays, multiplexing of rapid tests is becoming fairly common. Lateral flow tests, which are composed of a nitrocellulose membrane, sample pad, conjugate pad, wicking or absorbent pad and backing pad, are often challenging in their current format even though they are low-cost, lightweight and portable and require minimum sample preparation [69]. A common misperception of the lateral flow format is that it is neither quantitative nor able to be multiplexed. The absorbent pad provides a driving force based on capillary action, and the backing pad provides mechanical support to the device. Nitrocellulose membranes are the most popular and important materials in LFAs because they provide a platform for both reaction and detection during the assay. Capturing molecules, such as antibodies, can be deposited on the nitrocellulose membranes to form test and control lines via electrostatic interactions, hydrogen bonds and/or hydrophobic forces. Each set of two adjacent components overlap slightly to coordinate the fluid flow. Although challenging, particularly on a manufacturing scale, multiplexed, quantitative, and reader-based systems (or any combination thereof) are already on the market.

Since the 1990s, PDMS-based microfluidics has made little progress towards commercialization [70]; the developed products do not meet the needs of the end

users, who require chips and useful items [71]. Capillary-based microfluidics could be the solution for this problem [72], and currently, a suitable material must be developed for use in chips and expected biomedical microfluidics products [73]. As I described in Chapter 2, successful paper-based microfluidic devices could yield cheap, disposable, and portable diagnostic tests that are sensitive and specialized by taking advantage of the natural movement of liquid through paper without other functional units, especially pumps and syringes, which can be difficult to clean. The field of microfluidics addresses the precise manipulation of tiny quantities of liquid to minimize the quantity of the required chemical reagents to reduce the manufacturing cost. In addition to making the devices cheaper, the designers considered other characteristics that would make them more practical in the developing world. The test is lightweight and resistant to breaking, rendering it more portable than others, including those with glass surfaces. The paper chip is also easily disposable by incineration, a key consideration in both developing and developed countries concerned with maintaining public health. Moreover, the ability to direct the samples via multiple channels to different regions of the paper enables the detection of multiple biomarkers. Above all, such devices provide end users with an ideal preliminary screening tool for healthcare and are simple, inexpensive and useful [74]. However, paper-based diagnostics need to be further improved in terms of their clinical performance. As reported, existing paper-based analytical devices show varying specificity and sensitivity, which may cause false-negative or false-positive results. Another disadvantage of paper tests is their limited pumping action; they probably will not be able to perform more complex chemical reactions that the normal LOC can.

Instead of etching channels into a material, the network of channels inherent in paper can be used; these channels are patterned over the whole volume of the substrate, forming a capillary network. Moreover, the barrier of the selected channels can be made to be hydrophilic. Paper can also be treated with a hydrophobic polymer that directs the liquid along prescribed channels. Once the liquid reaches the wells at the ends of the channels, it interacts with reagents and turns the paper different colours. The colours can be matched to those on a colour key, much as they are in a pH test. A smart paper-based LOC could be better than normal microfluidic devices: coulometric reactions on paper replace sophisticated sensors and analysers, whereas using the paper's natural capillary action to absorb liquids avoids the need for external pumps or power sources.

4.2 Initial experiments with strips

4.2.1 Effect of cross-talk between samples

The strips were immersed in 1 ppm nitrite standard solution for 2 s and 10 s in the lab, and the results were compared to determine whether there is cross-talk among the pads of the commercial strip.

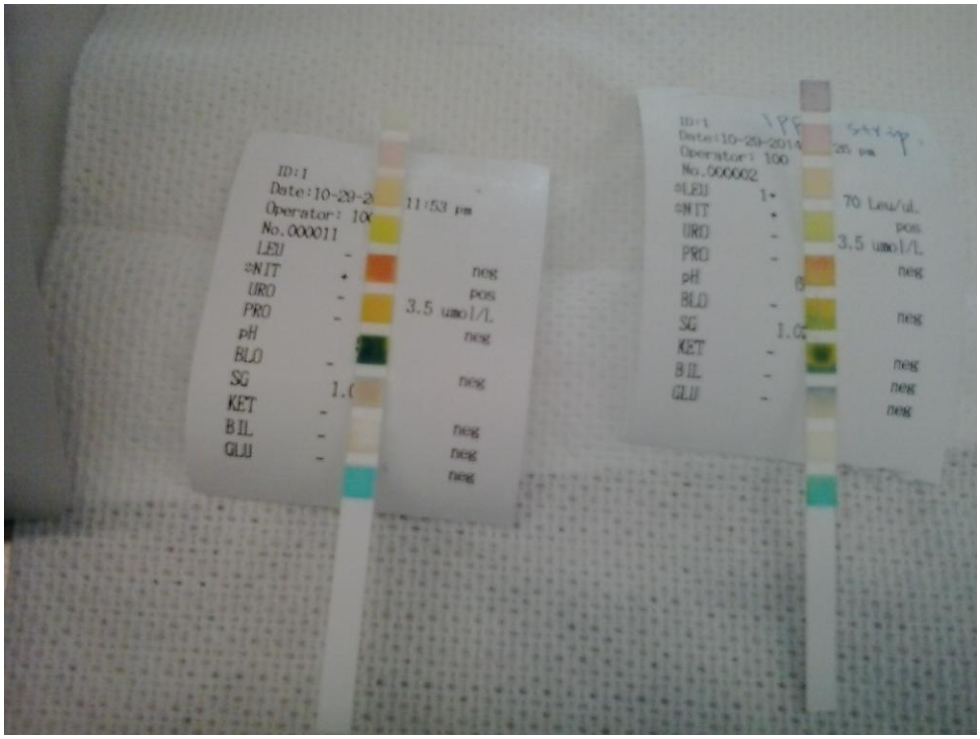


Figure 4.1 Comparison of the strips immersed in 1ppm nitrite standard solution for 2 s and 10 s

As we can see from Figure 4.1, the LEU value increase from 0 to 70 Leu/ μL; PH increases from 5.5 to 6.0; and SG increases from 1.005 to 1.020. We can thus conclude that diffusion among parameters should be avoided, so we should try to keep different biomarkers separated when design the paper-based lab-on-chip

4.2.3 Investigation of time-dependent characteristics of urinalysis colourimetric reactions

Studies on the changes of colourimetric reactions on normal urine strips within 60 min were performed using a urinalysis analyser.

The results obtained for the urine strips might change over time. The change in nitrite and pH can be neglected within 1 hour. The leukocyte count and the specific gravity both changed quickly. The machine could not read out the values for dry strips.

To compare the appearance of used urine strips within 24 hours, experiments were conducted, and their results are shown as Figure 4.2: The results prove that for NIT, pH and KET, the degree of saturation is reduced over time, but they keep the original colour, the reason of this could be oxidation. During the period of 7-9 h, we dried the strips deliberately, and the colours turned lighter than before, particularly for BLO (blood) and SG (specific gravity). When the dried strips became wet again, the saturation recovered.

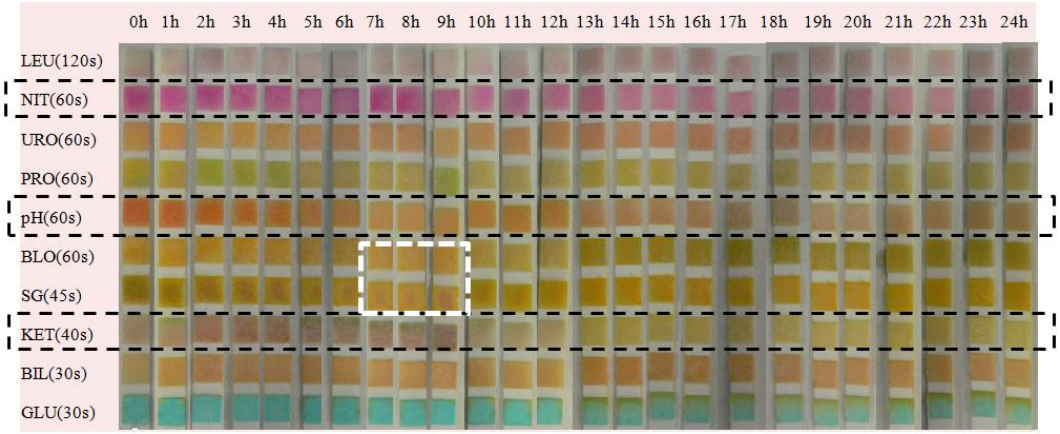


Figure 4.2 The appearance of used urine strips over 24 hours

Therefore, we can draw the conclusion it is important to keep the pads covered and wet to make the results validated for a long duration.

4.3 Design for diaper use

4.3.1 Surface layer of the diaper

The surface layer is a non-woven sheet that forms a liquid-permeable top layer that comes in contact with skin and is hydrophilic, breathable, soft, eco-friendly and comfortable. The layer gives the feeling of being dry and allows the urine to pass quickly into the absorbent core, thus keeping the user's skin dry, which vastly improves the integrity of the skin and inhibits bacterial growth. Commercial diapers always use a smart combination of tissue structures with superabsorbent polymers, which draws the moisture away from the skin and neutralizes the pH of the urine to avoid the development of UTIs.

4.3.2 Reaction layer

The reaction layer works with the help of capillary-forced lateral flow for transportation and the normal dipsticks' reaction after preparing each channel, which can provide colourimetric reactions for the detection of multiple UTI biomarkers. In this application of a paper-based LOC, the urine sample was placed in the middle of

the chip and then simultaneously flowed through the different channels via capillary action. After reaching the different wells in each channel, the colourimetric reactions occurred, and every well housed independent chemical reactions for detecting UTIs (Figure 4.3).

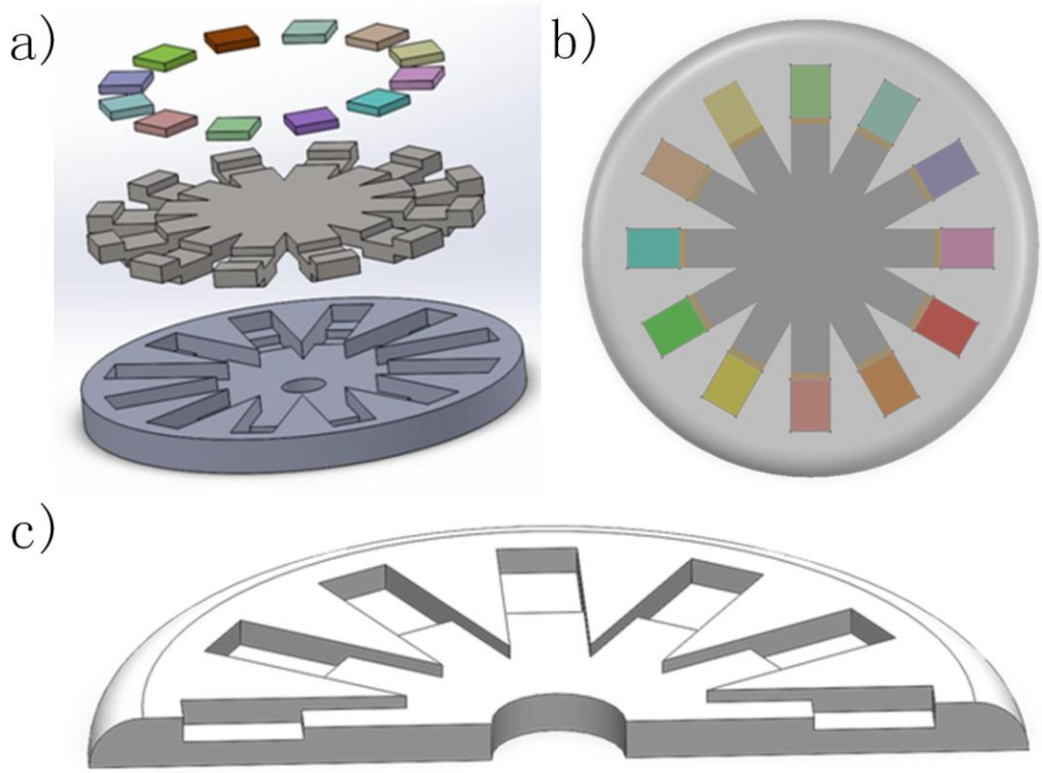


Figure 4.3 Arrangement of the reaction layer. a) the structure of the layers; b) the respected final product; c) the substrate

The fourth layer is one transport layer that works as both the detection window and provides the glue on the diaper needed for wear, which should be made of clear, flexible, transparent or translucent material suitable for numerous packaging. For example, material of PET (Poly Ethylene Terephthalate).

4.3.3 Exam the design in lab

To check if the reaction layer can work or not, The PDMS module was fabricated using the laser cutting machine as the substrate as illustrated by the design in Figure 4.3, and filter paper was placed at the bottom of each channel. The 10 ppm nitrite solution was used to test this method, and the results were as good as those presented in Figure 4.4.

Different nitrite solutions were detected using the above process. The results are shown below: the higher the nitrite concentration, the pinker the colour.

. Even though the PDMS is quite different as the Substrate I mentioned above which are suitable to wear, however, the functional to using separated channels to isolate the reaction is the same.

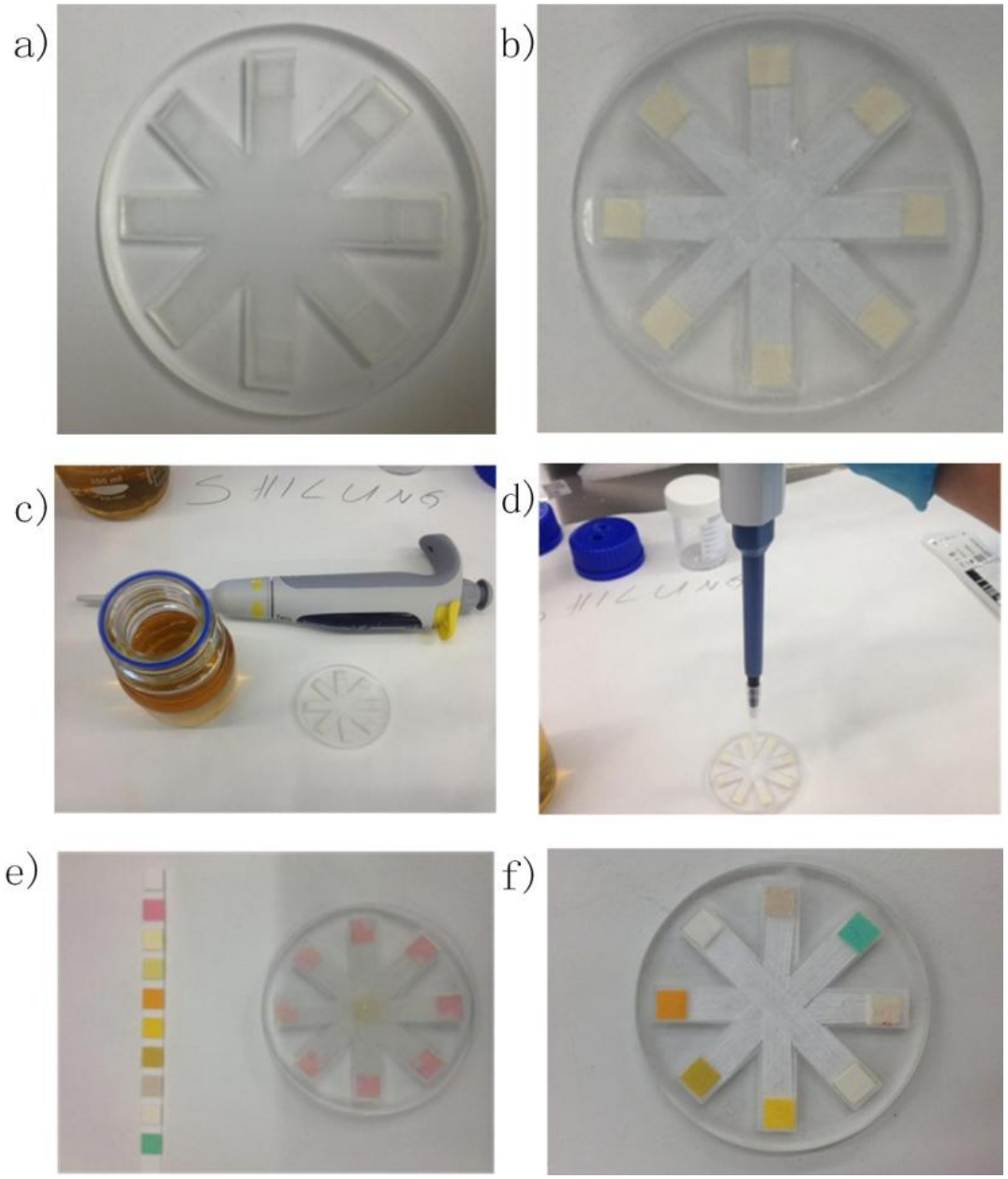


Figure 4.4 Test the feasibility of the design for reaction layer. a) model fabricated by laser cutting machine; b)put the filter papar at the bottom and pads at the end of every channel; c) the artificial urine with 10ppm nitrite was made; d) inject the sample in the middle as the design; e) the results is acceptable; d) experimental setup for other biomarkers as well.

4.3.4 Fabrication methods to make channels or wells

In this section, fabrication methods to make the specialized channels for transporting the biological samples and reactions areas for the coulometric reactions were investigated and fabricated in the BioMEMS lab. First, methods to render paper hydrophobic were investigated; based on these methods, hydrophobic barriers for the channels were created, and the samples could be transported to the specialized

reactions in the specialized wells. Second, wax fabrication methods were chosen based on their advantages and their ease of performance in the lab. Other fabrication methods were also investigated. Ways to make channels or wells on paper were also investigated because there will be a reaction pad in each well, which should also be considered. Third, a theoretical study was performed to analyse urine transportation; this study was necessary to ensure that the capillary force and paper characteristics did not introduce differences in transportation and detection in each well. Both the wet-out and fully-wetted flow conditions were studied and could be met.

There are mainly two methods for fabricating the microchannels and wells, one is cutting or engraving microfludic devices using a craft-cutting tool such as balde, which can make the channels as small as 45μ l. Another method will be embossing or cut-and-stack methods.

4.4 Theoretical study

There were fundamental and important issues to evaluate: controlling the effect of rough channels on sample transport, enabling the samples to reach the reaction wells at the same time, achieving the aim of simultaneously detecting multiple biomarkers, and providing experimental information on the fabrication and detection. Capillary action was used to provide a force similar to a pump or syringe; capillary action is driven by characteristics such as hydrophilicity and surface tension and porosity. The situations for transporting liquid can be divided into two: one, when samples first contact the dry strips, which is an important situation because it determines whether the channel is adequately fabricated for sample transportation as well as whether the signals can be simultaneously detected in a multifactorial manner; and two, the detection of liquid flow on a previously wetted substrate. Both of the situations must have an equal ability to detect multiple biomarkers in multiple channels.

4.4.1 Paper wet-out

When a sample is added in the middle of the designed device, the samples will be transported at the same time to the wells. To examine these situations, Washburn's equation was used, which illustrates that the distance moved by the fluid front is proportional to the square root of time (Equation (5)) [76]

$$L = \sqrt{\frac{\gamma D t}{4\eta}} \tag{5}$$

Here, L is the distance covered by the fluid front, t is the time, D is the average

pore diameter, c is the effective surface tension, and g is the viscosity. The liquid transportation rate can be determined by the characteristics of the surface, the impregnation of hydrophilic/hydrophobic barriers and the channel width.

4.4.2 Fully-wetted flow

In the other situation, which is based on a wetted substrate, the function of adding additional sample could be represented by continuous pee, which can maintain the humidity; therefore, we should have an understanding of this situation as well. Here, Darcy's law (1856) was used, which is based on the Navier–Stokes equations by assuming homogeneity [77,78] and is described as:

$$Q = -\frac{\kappa WH}{\eta L}\Delta P \tag{6}$$

where Q is the volumetric flow rate, k is the fluid permeability of the paper, g is the viscosity, WH is the area of the channel perpendicular to the flow and DP is the pressure difference along the flow direction over the length L.

4.5 Wax-based fabrication

Due to the design from Dr. Nuno Pires, Dr. Zhong Fang and Master student Chaohao Chen, fabricating the hydrophobic channel and hydrophilic barriers on one filter paper is needed. To do this, different fabrication methods were investigated and Wax-based fabrication was tried in the bioMEMS lab. Firstly, Methods to render paper hydrophobic was investigated, since based on the methods, hydrophobic barriers for the channels could be made, and sample can be transported on the specialized reactions to the specialized wells. Secondly, wax fabrication methods were chosen to be investigated since their advantages and easy to be done in the lab. Other fabrication methods was also investigated.

4.5.1 Methods to render paper hydrophobic

Impregnation with wax prevents cross-contamination of reaction zones in dipstick assays. This method can be dated back to 1902. As an alternative to forming hydrophobic barriers, test device carrier matrices composed of absorbent under-layers were fabricated to prevent cross-over from one matrix to another. Another alternative method involved separating the reagent area into different places. There are many ways to render paper hydrophobic and thereby enable the walls of the channels to keep the samples inside the channels, including spraying alcohol suspensions of SiO2 nanoparticles onto the paper surface [81], soaking in polystyrene solutions,

photolithography patterning with SU-8, wax printing, plasma processing, and treating with silanizing reagents.

4.5.2 Wax fabrication methods.

Currently, it is widely acceptable to use wax-based fabrication methods because the resources are easy to obtain and the methods are cheap and easy to operate, can be completed in 5-10 min, and do not require a clean room, UV lamp and toxicant reagents. There are 3 main methods: (i) screen printing, (ii) dipping and (iii) printing.

In wax screen printing, solid wax is rubbed through a screen onto a paper sheet, and the paper is placed on a hot plate (or oven) to allow the wax to melt and diffuse into the paper, thus forming hydrophobic barriers [82]. Wax dipping (Figure 6B) [83] requires a mould that can be formed by laser cutting. The pattern can be transferred onto a piece of paper by dipping an assembled mould (sealed with magnets) into molten wax. The optimum temperature and dipping time are 120-130℃ and 1 s, respectively. Although both wax dipping and screen printing do not require high-cost fabrication equipment, they suffer from inflexibility in patterning and low reproducibility between batches. There are 3 main methods for wax printing: (i) painting with a wax pen, (ii) printing with an inkjet printer followed by painting with a wax pen, and (iii) directly printing with a wax printer.

As in the case of modelling the fluid movement, Washburn's equation can be used to predict the spreading of molten wax and the final width of the hydrophobic barriers in paper substrates as equation 2[84], where L is the distance covered by the wax front, g is the viscosity, c is the effective surface tension, D is the average pore diameter and t is the time.

Notably, the viscosity of molten wax is a function of time and exposed heat. Therefore, the characteristics of the final hydrophobic barriers that are formed in a paper substrate are defined by the initial deposited wax. The inner width of the hydrophobic channel can be defined as Equation (7), as follows:

$$W_C = W_P - 2L \quad (7)$$

where W_C is the inner width of the hydrophobic channel, W_P is the inner width of the printed channel, and L is the additional distance that the wax spreads perpendicular to the length of the channel. Here, L is a function of time, heat and the porosity of the paper.

4.5.2.1 Wax screen printing

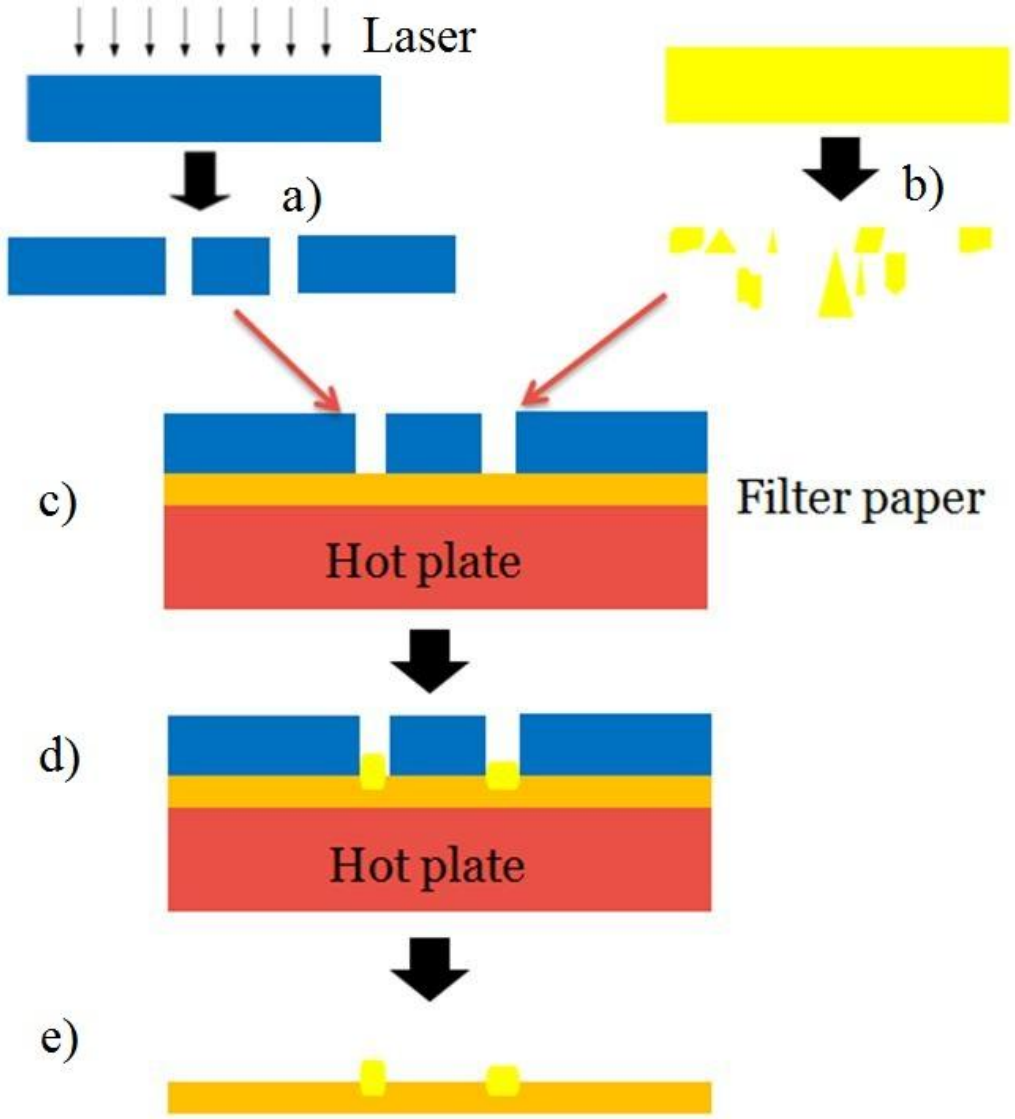


Figure 4.5 Fabricating process of wax screen printing. a)Use laser engraving equipment to fabricate Print moudle . Then clean it use ultrasonic. B) rub the wax to be small pieces .c)Use the rubbed wax to structure of Print moudle.d)Use the hot plate to bake the filter paper. So that the wax will infiltration the fiber in the filter paper. f) Then we get the wax channel on the paper.

The fabricate process of Wax screen printing is shown as above, as the Figure 4.5.

4.5.2.2 Wax dipping

4.5.2.2.1 Fabrication process

The fabrication process of wax dipping is shown as below, as the Figure. 4.6

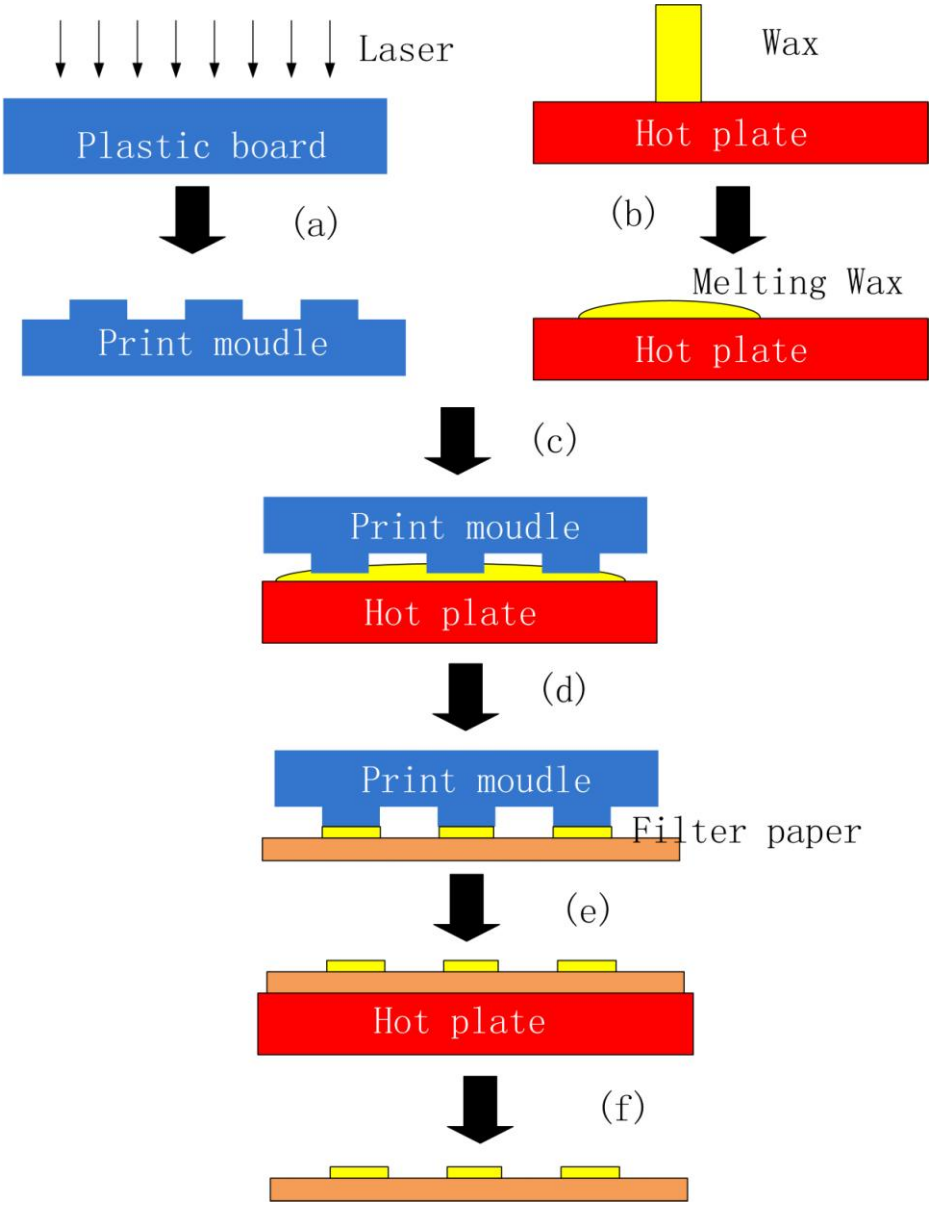


Figure 4.6 Fabricating process of wax screen dipping. a)Use laser engraving equipment to fabricate Print moudle . Then clean it use ultrasonic. B)Use hot plate to melting the wax .c)Use the melting wax to infiltration the surface of Print moudle.d)Print the wax to the Filter paper surface.e)Use the hot plate to bake the filter paper. So that the wax will infiltration the fiber in the filter paper. f) Then we get the wax channel on the paper.

4.5.2.2.2 Method perform in the lab

Figure 4.7 Realization the fabrication process in the lab. a) the module fabricated by the laser cutting machine; b) the baker; c) the final made product.

The wax dipping was done in the lab, module was fabricated by the laser cutting machine as Figure 4.7a) shows, the baker in Figure 4.7 b) was worked as the hot plate. While Figure 4.7c) shows the fabrication results done in the lab.

4.6 Experiments of wax-based fabricated product in bioMEMS lab

4.6.1 Executing the design in the lab

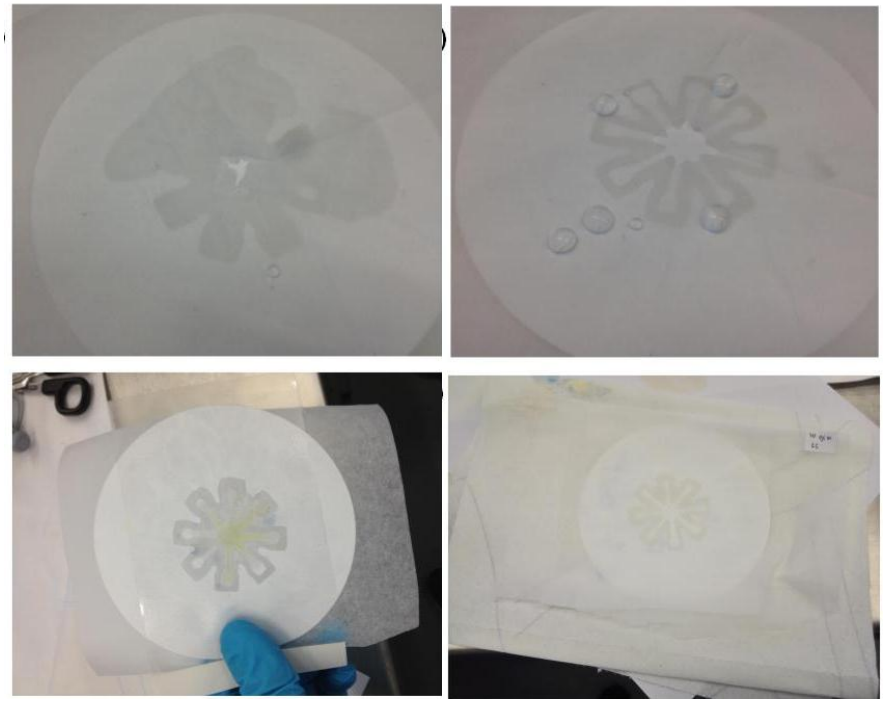


Figure 4.8 Executing the design in the lab. a) The results when samples were dropped onto the hydrophobic paper layer or b) the first surface layer. c) The function of the surface layer, which can keep the top dry.d) The transport bottom layer, which is the transparent window for detection.

Based on the fabricated product shown in Figure 4.7c), execution of the design was done in the lab.

4.6.2 Examination of the fabricated Reaction layer

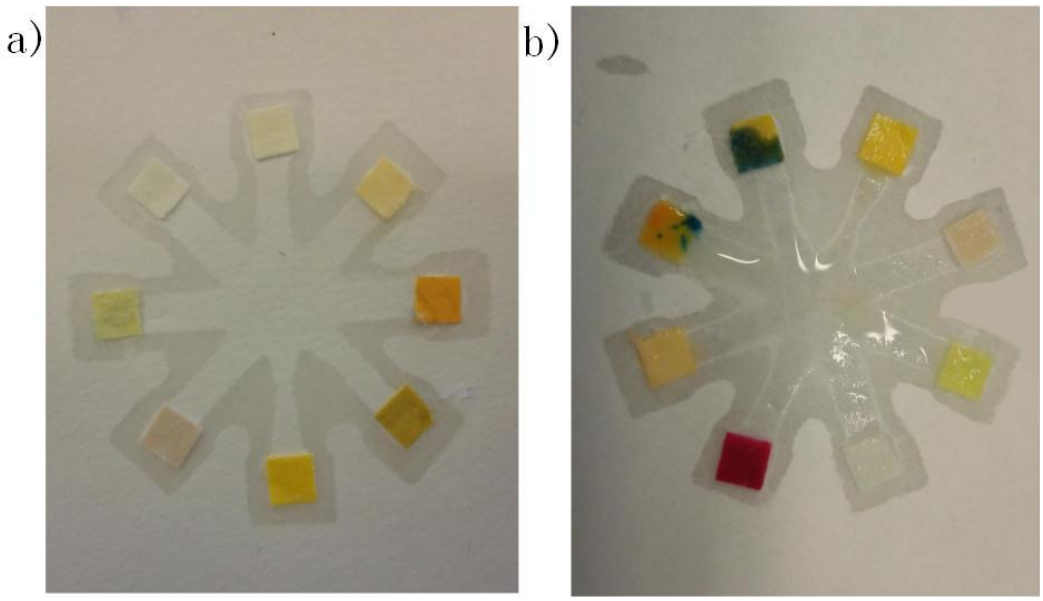


Figure 4.9 Reaction on pad. a) the pads were assembled onto the end of every channel of reaction; b) aritificial urine was used to test the product.

Different pads from the commercial normal strips were cut out and assembled onto end of each channel as Figure 4.9 a), artificial urine mixed with nitrite was used to test the product, which shows the channels were well formed by the wax hydrophobic barriers and the nitrite pad turn to be red, which proves the feasibility to detect the biomarker of nitrite, thereby, showing the feasibility of detecting UTI.

4.6.3 Unchanged channels when the chip is folded during wear;

When wearing the diaper, one cannot avoid folding the chip; however, our smart design utilizes capillary force that is not affected by wearing. Bromophenol blue solution was used to test the fabricated product, as Figure 4.10a). The results are presented as below (Figure 4.10b).

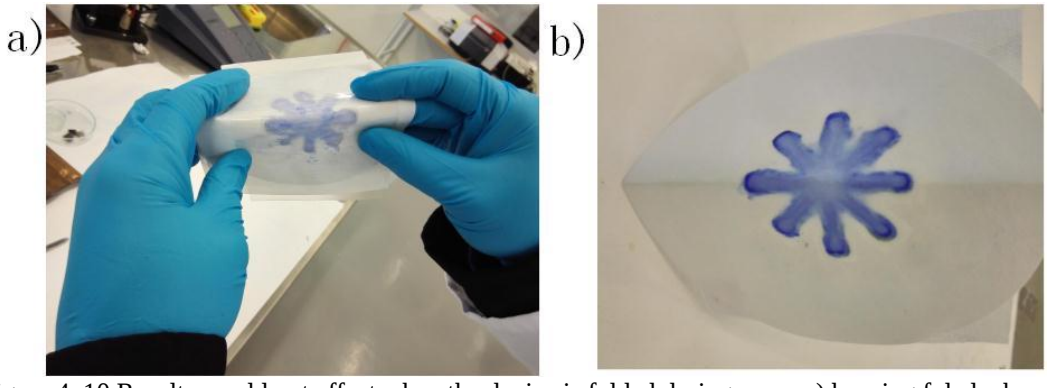


Figure 4. 10 Results would not affect when the device is folded during wear. a) keeping foled when doing the experiment; b) the optimal result.

4.6.4 The results can be validated in 24 hours.

After 24 hours, the colour of the samples did not change, which demonstrated that this device can preserve the sample for 24 hours.

4.7 Fabricate in factory

4.7.1 Sample preparation in the factory

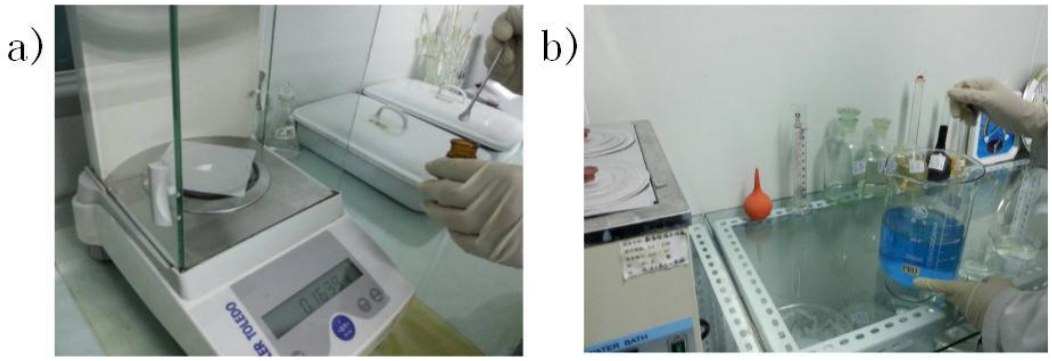


Figure 4.5 sample preparation in factory. a) weight the components; b) control the ratio of components.

Above Figure 4.11 is for the sample preparation in the factory, the weight and ratio of the components for making the biomarkers was strongly controlled.

4.7.2 Biomarkers synthesis

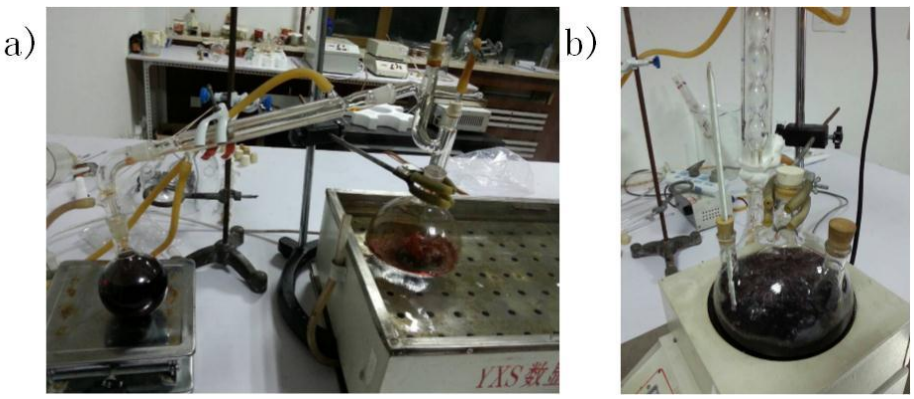


Figure 4.6 Synthesis the biomarker. The biomarkers were made by several chemical processes.

The biomarkers are synthesized by depression distil for a long period and it needs many processes.

4.7.3 Manual strips-making

The strips will be impregnated firstly, and then be dried, which were shown as Figure 4.13).

a)  b) 

Figure 4.13 Manual strips making. a). impregnation; b) dry the strip.

4.7.4 Machinery strips-making

The principle of machinery is the same as the manual strip-making, shown as Figure 4.14.

a)  b) 

Figure 4.7 Machinery strips making. a). impregnation; b) dry the strip.

4.7.5 Strips pads making

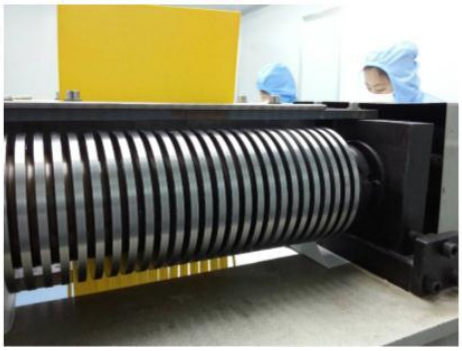
a)  b) 

Figure 4.8 Strips . a) the whole paper are cut into the constant width of channels; b) the storage of the cut strips

The whole paper will be cut by machine shown as Figure 4.15 a) to the constant widths of strips as Figure 4.15b). and then they will be cut as pads when needed.

4.7.5 Assemble the pads on paper

The fabricated pads were assembled onto the Whatman filter paper, as Figure 4.16 shows.

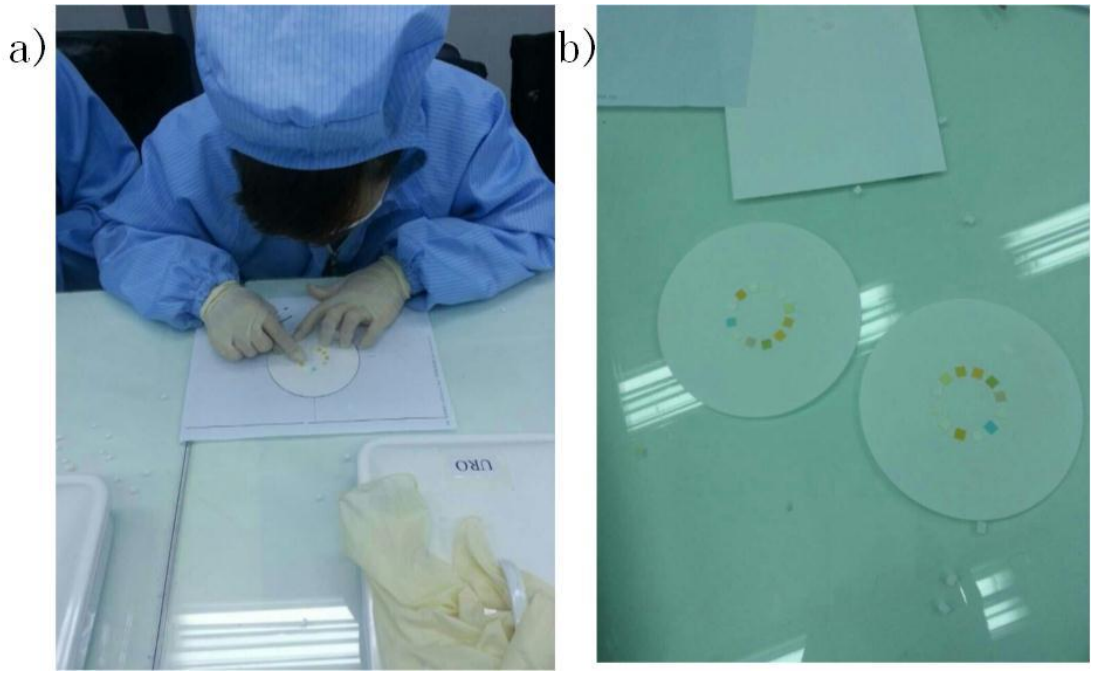


Figure 4.9 Assmble the pads on device. a) working process; b) finally product.

After this, the product will be done by the wax dipping fabrication, which I showed in Figure 4.6 and Figure 4.7. After fabrication the channels, the whole assemble will be done as Figure 4.8. Further fabrication will be done.

4.8 Introduction of existing detection techniques to the paper platform and exploration of extracting quantitative readout via handhold devices and camera phones

For a qualitative detection, colourimetric changes in paper-based diagnostic assays can be visualized by the naked eye to yield a yes/no answer. In contrast, imaging analysis by a handheld reader or a cell phone[85] and visual estimation by the naked eye (by comparing the colour change with a predetermined score chart) [86] are used to report quantitative readouts. However, both qualitative and quantitative strategies suffer from low sensitivity and poor accuracy. Additionally, the performance of paper-based diagnostics can be enhanced by reducing their complexity and increasing their functionality, such as the integration of sample pre-treatment and result analysis. Digital colour analysis is a useful tool for obtaining more quantitative results compared with the simple visual inspection of colour changes.

To optimize the device for developing countries, it would be useful to combine the paper tests with a system of cell phones for off-site diagnosis, thereby minimizing the level of expertise needed to use the tests, which is a way of conserving the valuable time and limited resources of health care providers in rural areas where doctors are limited. Thus, people who are trained to conduct the tests can perform them and send them to a central facility, where a doctor can diagnose and treat the

patients without having to actually be present. It is a good idea to use the two low-cost systems of paper-based fluidics and cell phones, which are quite ubiquitous in the developing world.

Conclusion and future work

Possible solutions for the rapid screening of UTIs on diapers were investigated and developed. After extracting urine from diapers, the samples were read using an existing LOC, which was not sensitive enough and required multiple steps. The biomarker was not sensitive enough, but there were advantages to detecting UTIs with an LOC. Electrochemical sensors are real-time, selective, sensitive, non-reagent consuming, and quantitative but are complex, fragile, and susceptible to pH, ionic strength, and temperature. Importantly, there was not enough time to create an array for the detection of multiple biomarkers. Finally, colourimetric reactions on diapers were chosen, which are rapid, cost effective, and feasible; require few steps; and have a verified methodology. Preliminary experiments on normal strips and the diapers were performed. Paper-based microfluidic LOC POC diagnostics and wax-based fabrication methods were investigated, and simple tests were performed in the lab to determine an appropriate design for applying these techniques on a diaper.

Paper-based microfluidic devices have been widely employed to develop POC diagnostics due to their low cost and easy scale-up in manufacturing. With the integration of materials science and biomedical engineering, paper-based diagnostics are becoming simpler, more sensitive, more accurate, and multi-functional. One task is to combine paper diagnostics with mobile phone-based optical detection to utilize telemedicine detection to improve health-care services in resource-limited settings [168]. The second task could be to fabricate more precise devices, and additional tests will be conducted in the future with the help of theoretical simulations and optimizations, thereby generating a reliable biomedical microfluidics product. The clinical improvement in the final paper-based microfluidic devices, the validation of the quantitative readout of the results, and the usage of these devices directly on diapers will be considered in future work.

Ending

After I chose the project -Sampling module and biological protocals of touch-screen based biosensor for detecting UTIs, I begin to do the biomedical investigations on the UTI. At that time, various detection methods and biomarkers were searched and tried.

Sampling module was tried, I had done the experiments to prove that the strong absorption of the diaper and the solutions were also been found which was injecting the salt water to release the urine out. Of course, there are many biomarkers and methods tried in my group, but totally I had tried 2 methods by myself, and partly involved in the third method.

The first trail for the detecting UTI was existed lab-on-chip bioluminescence detection, one existed chip was designed and fabricated by Dr. Xinyan Zhao from HBV and COMSOL simulation and research studies were done on my new chip design, finally, detecting UTI on chip was realized on this chip with the help of PMT， which is accurate and novel, however, we gave up this method since the biomarker ATP cannot provide the sensitive and quantitative quick detection.

Since I turned to focus on the second method-electrochemical sensor which could detect nitrite quantitatively and accurately, after building the relationship between the nitrite concentration and number of E.coli, we build the relationship between the UTI possibility and the value shown on the screen, however, we gave up at the final stage as it is easily affected by temperature; expensive and easy to be broken, more importantly, only one biomarker cannot provide the reliable result and I cannot finish the electrochemical array to multi-detect several biomarkers within the short period of project.

Based on the two methods tried, I was attempting to find a single, easy method that is useful, cheap and convenient. I found the paper-based lab-on-chip method that combines lateral flow and normal strip detection could be the solution. Numerical investigations were done on the paper, lateral flow, paper-based assays and theoretical studies. This method can detect the urine sample directly on the diaper without sampling and preparation. I then design the chip which is curving or embossing channel on the paper and exam the feasibility in the lab. Before I want to continue, Dr. nuno Pires, Dr. Haakon Karlsen and Master student Jennifer Panugan and Chaohao Chen made a new design which can form the channel directly on the paper by making the hydrophilic barriers, then I focus on the Wax-based fabrication, where investigations and experiments were done, and simple practical tests were done including if the hydrophilic barriers can form the channel or not, weather the generated fold will affect the result and whether the device can keep the result be validated till 24 hours. The results were as good as expected. I also went to the factory who can make the paper-based assays, there I learnt the sample preparation, the Synthesis of the biomarkers process, manual and machinery strips making process, which builds the foundation for us to realise our design

and fabrication. The further work including the final fabrication under the medical requriments and the readout which can provide the quantitative results and send to nurses or patients easily.

Then, biological protocols of touch-screen based biosensor for detecting UTI would be generated and one device which is quite cheap and useful would be made, and the UTI can be detected out from diaper, and the nurse would never be worried about extracting urine from patients(old or disabled people), they will get the satisfied solution.

After I chose this project, "Sampling modules and biological protocols for touch screen-based biosensors for detecting UTIs," I undertook biomedical investigations on UTIs. At that time, various detection methods and biomarkers were researched and tested.

Sampling modules were tested; we had performed the experiments to demonstrate the strong absorption by the diaper and had identified the salt water solutions that could be injected to release urine.

The first trial for detecting UTIs utilized existing LOC bioluminescence detection; one chip was designed and fabricated by Dr. Xinyan Zhao from HBV, and simulations and research studies were performed. Ultimately, we were able to detect a UTI using this chip with the help of PMT, which is accurate and novel, but it was difficult to create the contact surface, so we abandoned this method.

Then, we focused on the second method, an electrochemical sensor, which could detect nitrite both quantitatively and accurately. After elucidating the correlation between nitrite concentration and the number of *E. coli*, we built the relationship between the possibility of a UTI and the value shown on the screen; however, we relinquished this idea at the final stage because it was easily affected by temperature, expensive and easy to break.

I was attempting to find a single, easy method that was useful, cheap and convenient. I identified the paper-based LOC method that combines lateral flow and normal strip detection. This method can analyse the urine sample directly on the diaper without sampling and preparation. The results were as good as expected.

Then, biological protocols for touch screen-based biosensors for detecting UTIs were generated, and one device that is quite cheap and useful was made: a UTI could be detected from a diaper, the nurse would never worry about extracting urine from patients (elderly or disabled people), and a satisfactory solution will be obtained.

Shilun Feng

28.05.2015.

Buskerud and Vestfold University College, Norway

Reference

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Bibliographic Summary

I have 4 publications already, which are:

a) S.L Feng, T. Dong, "Design and Characterization of a Lab-on-chip for Continuous Bioluminescent Measurements of ATP," 2014 IEEE International Symposium on Medical, Measurements and Applications (IEEE MeMeA 2014), Lisbon, Portugal, June 11-12 2014

b) S.L. Feng, Z.C. Yang and T. Dong, " Detection of Urinary Tract Infections on lab-on-chip device by measuring bioluminescent photons emitted from ATP," Conference Proceedings of the 36th Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC'14), Chicago, USA, 2014.

c) S.L Feng, T. Dong, " Applied Technology in Diaper-based UTI Testing for Elder People by Using Nitrite Ion Selective Electrode," 2014 2nd International Conference on Mechanical Engineering, Civil Engineering and Material Engineering (MECEM 2014), Wuhan, China, September 27-28,2014.

d) S.L Feng, L.E. Rosen and T. Dong, " Quantitative detection of Escherichia coli and measurement of urinary tract infection diagnosis possibility by use of a portable, handheld sensor," 2015 IEEE International Symposium on Medical, Measurements and Applications (IEEE MeMeA 2015), Torino, Italy, May 07-09, 2015.

Design and Characterization of a Lab-on-chip for Continuous Bioluminescent Measurements of ATP

Shilun Feng
Department of Micro- and Nanosystem Technology
Faculty of Engineering and Maritime Science
Høgskolen i Buskerud og Vestfold, HBV
Boks 2242, N-3103 Tonsberg, Norway
Shilun.feng@gmail.com

Tao Dong (*Corresponding Author*)
Department of Micro- and Nanosystem Technology
Faculty of Engineering and Maritime Science
Høgskolen i Buskerud og Vestfold, HBV
Boks 2242, N-3103 Tonsberg, Norway
Tao.Dong@hbv.no

***Abstract*—A novel multifunctional two-sides silicon lab-on-chip (LOC) was designed and fabricated for providing a high-efficient long-term continuous analysis platform of measuring the concentration of adenosine 5'-triphosphate (ATP) based on bioluminescence. This lab-on-chip mainly consists of Y shape internal ribbed micro mixers, T-junction droplet generators, different kinds of spiral time delay channels (TD-Cs) and the chambers. Enzyme solutions and ATP were separately injected into the chip at different flow rates through 3 inlet ports. Due to the advantage of Segmented Flow Analysis (SFA), it was introduced and applied with the T-junction droplet generators in this paper, and thus oxygen was injected into the central of the bottom surface through another main inlet periodically. Numerical investigations prove that combing with SFA and T-junction droplet generator is stable to transport the sample; micro mixer can achieve an outstanding mixing efficiency in a small space; the detection of ATP standard solution at optimal conditions was successful, keeping a good linearity between the concentration with the intensity of light during the range from 10^{-5} mol /ml to 10^{-9} mol/ml.**

Keywords—lab-on-chip; internal ribbed micro mixer; T-junction droplet generator; adenosine 5'-triphosphate (ATP).

I. INTRODUCTION

The multifunctional microfluidics-based biochip is promising. It can provide exciting possibilities for parallel immunoassays, high throughput sequencing, DNA sequencing, blood chemistry for clinical diagnostics, and environmental toxicity monitoring. It is said that complexity of microfluidic devices tend to be significant in the near future due to the need for multiple and concurrent biochemical assays on reconfigurable and multifunctional platform [1].

Rapid detections of cells in drinking water and food are of great importance to public hygiene and food safety. The bioluminescent assay method, using an ATP dependent-luciferin-luciferase reaction, is a reliable technique to measure biomolecules [2] in a sample or the number of cells [3,4]. And urinary tract infection, as the most common organ system to experience bacterial infections has also being using the ATP Bioluminescent Measurements [4]. The mechanism of the bioluminescent reaction occurs as follows [5]:

$$\text{ATP} + \text{luciferin} + O_2 + \text{luciferase} \rightarrow \text{AMP} + \text{oxyluciferin} + CO_2 + \text{PPi} + \text{hv} \quad (1)$$

Luciferase produces cold light (~560 nm) by the ATP-dependent oxidation of d-luciferin. Consequentially, the amount of ATP is directly associated with the amount of emitted light by luciferase, which in turn represents the amount of cells in the solution. Despite this, the poor stability and high cost of luciferase and d-luciferin in bioluminescent reactions prevent the extensive usage.

A promising alternative is to manipulate liquids as discrete droplets, based on the principle of continuous fluid flow [6]. SFA, also namely Segmented Flow Analysis, use nitrogen bubbles or air bubbles to separate the sample into short fragments and manipulating liquids as discrete. It can make the process automatically and stably, which can be helpful for the online continuous system, by controlling the time and velocity of the samples and reagents traveling in the chip. Specially, it can also guarantee sufficient oxygen supplying for the cells in droplets.

In this paper, based on one chip designed which is a continuous sensing of acute toxicants in Drinking Water [7], a updated silicon chip composed of four groups of Y shape Internal ribbed micro mixer, T-conjunction droplet generator, TD-C and chambers was designed and fabricated. Several investigations had been done on the T-junction droplet generator, Y shape internal ribbed micro mixer and measurement of ATP.

The four micro mixers recreate optimal mixture conditions for the sample, sensor cells and buffer solution in the top side while the T-junction droplet generator encapsulates them inside aqueous droplets separated by air. The droplet flow was investigated through numerical simulations since it represents an innovative two-phase flow that allows to oxygenate living cells and to operate at lower pressure than the common two-phase flow [8].The mixed solutions are drove by the O_2 form the central utilizing SFA to the different TD-Cs which provides

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the time-consuming platforms for Fluorescence. The concentration of toxicants in water can be measured through a small observation chamber and a penetrated chamber with fixed volumes, and then light intensity of the observation chamber is measured on time. The photomultiplier tube (PMT) based detection module will be also installed for quantify weak signals of the bioluminescence reaction (quorum sensing) [9].

II. EXPERIMENTAL SECTION

A. Chip design and configuration

This is a novel silicon chip with two sides which mainly has four groups of Y shape internal ribbed micro mixer, T-junction droplet generator, TD-C and chambers.

For the top side, internal ribbed micro mixers which inherit the design of micro-concentrator to force two fluids to flow in the same internal ribbed channel, improving the mixing efficiency. The micro mixers designed has successfully made three solutions mixed. For the bottom side, O_2 will go through the main inlet firstly to the center and disperse into 4 channels at the same velocity. With the help of T-junction droplet generator and SFA, the encapsulated droplet will be made and brought from the bottom side to the top side, after which it will be broken up and transported to the different TD-Cs at control time and velocity..

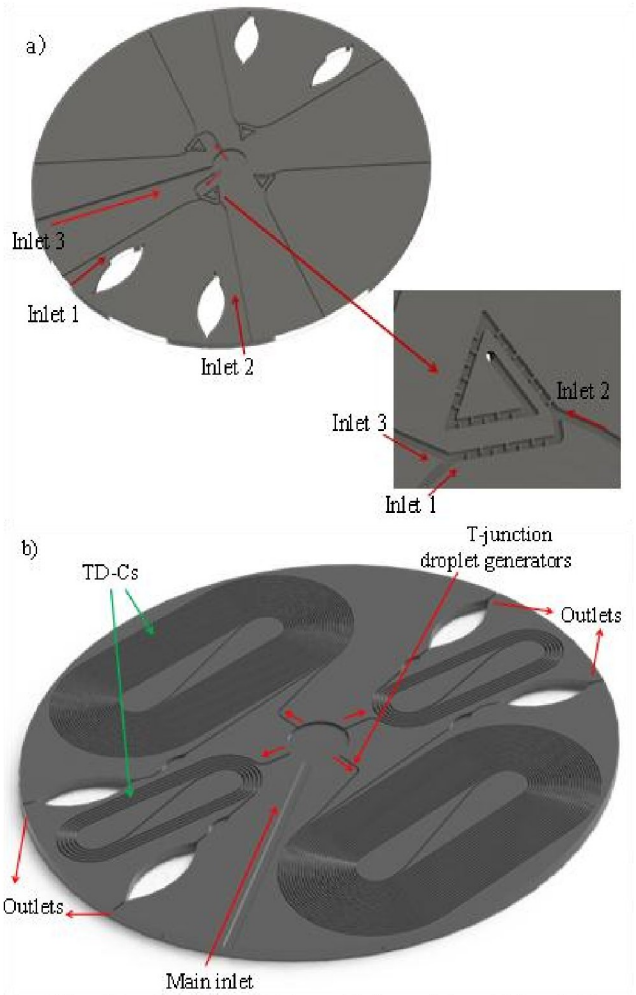

Fig. 1 Novel Double-sided multichannel living-cell-based Microfluidics chip; (a) top side with the structure of the mixer; (b) bottom side

B. Fabrication

Both sides of the 4-inch chip were fabricated using a previously-done silicon micromachining process [10]. The processing flow is shown in Fig. 2.

C. T-conjuction droplet generator

Fig. 3 shows schematically the geometry of the T-junction. The continuous mixed solutions pass through the channel on the top side of the chip and after an orthogonal channel, merge with the bubbles from the center in the channel on the bottom side.

The droplet creation, cutting and transporting had been investigated for the further valuable meaning [11]. The mixed fluids form an interface at the T junction of the penetrated hole and main channel on the bottom side. The discontinuous phase stream penetrates into the main channel and a droplet begins to grow; the gas from the center made by SFA in the main channel distorts the droplet into the downstream direction. Resulting in the interface on the upstream side of the droplet moves downstream and the droplet break appears at the neck connecting the punched hole and main channel. The disconnected liquid droplets flow downstream in the main channel, while the tip of the stream of the discontinuous phase retracts to the end of the T conjunction and the process repeats.

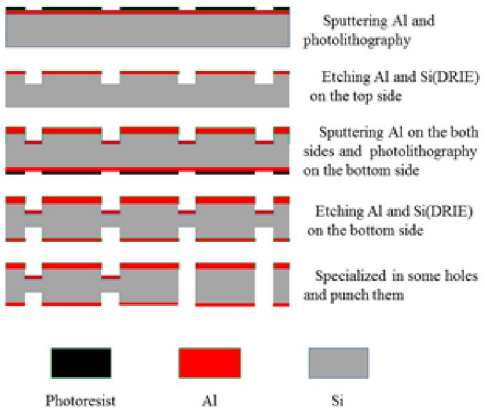

Fig. 2 Process flow of the cell-based LOC device

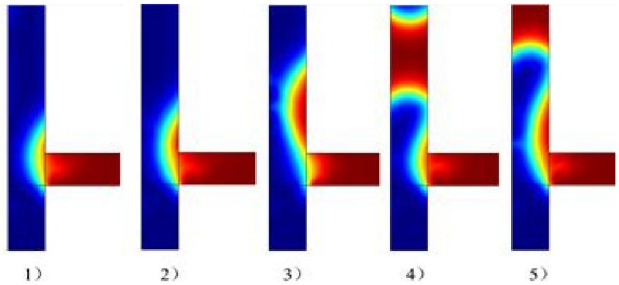

Fig. 3 Process to generate the droplet

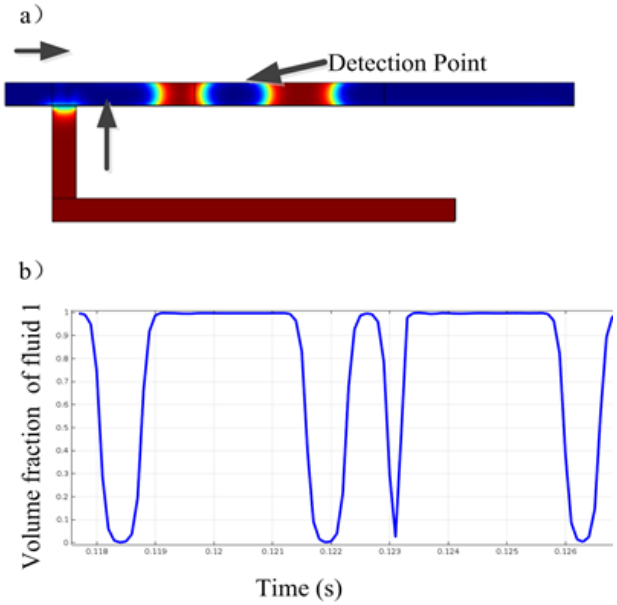

Fig. 4 Investigation of the T conjunction droplet generation; (a) structure of the conjunction generation; (b) volume fraction of fluid 1 changing with time

Finally, this process generates uniformly sized droplets (Fig. 3) and the volume of these droplets can be adjusted by changing the rates of flow.

We are interested in how the sizes of the droplets formed in a T-junction depend on the rates of flow, the viscosity of the continuous and discontinuous fluid (not studied here), and if these droplets can be continuous. So the detection point (0.025, 0.4) has been selected, relations between volume fraction and fluid with time has been drawn.

D. Y shape internal ribbed micromixers

Different mixers had been investigated for a long time [12], the Y shape internal ribbed micro mixer which have been proved its mixture efficiency had been used by me.

To make the three Reagents from the middle and two edges possible to be mixed, two Y shape mixers were used; to make the mixing totally in a small space and convenient to move them to another side, the structure in the fig. 5 a was designed. And simple COMSOL simulation of shear force distribution had been done to check its killing capacity to the cells.

E. Bioluminescent Detection tests of ATP

ATP and enzyme solutions were pre-treated by Multifunctional sample preparation kit [13] and injected into the microfluidic chip at different flow rates through 3 inlet ports separately (Luciferase goes through inlet 3 into mixer, ATP goes at inlet1 and luciferase goes at inlet 2). In optimized conditions the method will be successfully applied to measure the concentration of ATP by evaluating through the intensity of emitted light measured by the PMT-based detection module. After the observation chamber is filled with gas-liquid two-phase flow, the detection module starts to work. The luminescence data will be calculated using Microsoft Excel® and OriginPro® software.

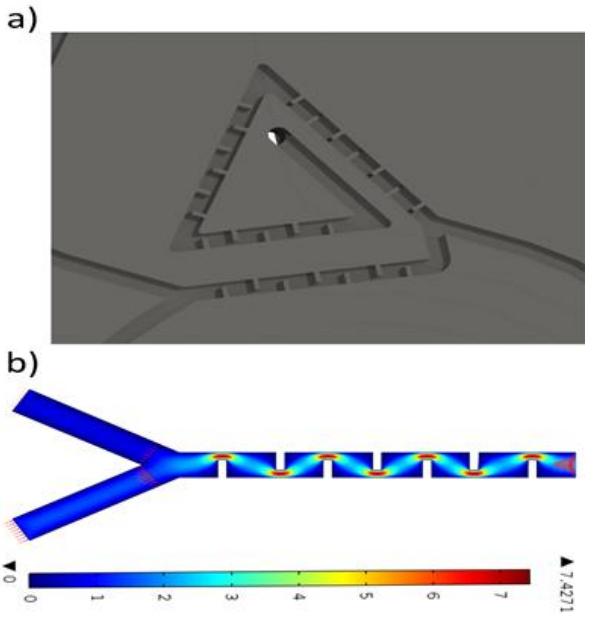

Fig. 5 Two-dimensional mixing properties of the counter-flow micro mixer; (a) structure of the mixer; (b) shear rate distribution around a single counter-flow unit

ATP Assay Kit (Merck Chemicals Ltd , United Kingdom) and enzyme solutions (Shanghai bioleaf® Biotechnology Co., Ltd. China) were used to test this chip.

III. RESULTS AND DISCUSSION

A. SFA used with the T conjunction droplet generator

T conjunction droplet generator is useful for not only encapsulating cell sensors to the droplets but also making a bridge to the bottom side to the top side. SFA plays a vital role for making droplets generated by the T conjunction moving periodically and continuously, which can make the system stable. It can be seen form the COMSOL simulation that the volume of liquid droplets and gas are almost periodic, therefore, the system is stable.

B. Y shape internal ribbed micromixers

The mixer had been designed as picture Fig. 5a shown, which was selected for reaching a biggest mixture effects comparing with some other mixers, so mixture simulation has been neglected. COMSOL simulation also had been done for the shear rate change when reagents meet with the rigs and the maximum shear force magnitude will not be harmful to the cells. The design is successful for it modifies one mixer made before (12) which has proven its mixture abilities and makes the possibility that mix reagents totally in the small space, finally making the droplet generator happening with the T junction droplet generator.

C. Bioluminescent Detection tests of ATP

When the ratio of ATP to enzyme solution was fixed, the optimum flow rates of enzyme, ATP, and enzyme solution was 3.6, 8.2, and 3.6μl/min, respectively. The optimal total flow

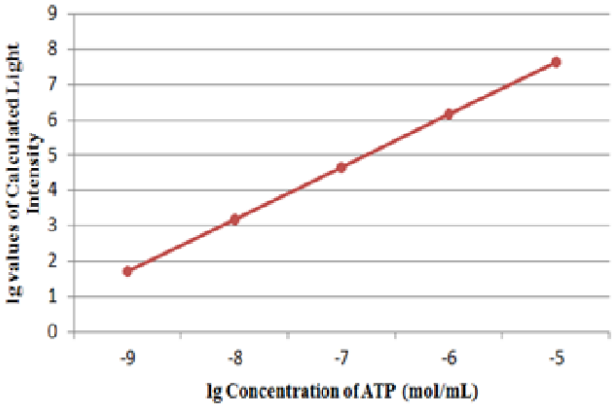

Fig. 6 the standard curve between ATP Concentration with calculated value of the light intensity.

rate was 15.2 μl /min.

ATP standard serials of solutions were made into the concentration 10^{-9}、10^{-8}、10^{-7}、10^{-6}、10^{-5}mol/ml respectively. With the help of the PMT detection, the standard curve of ATP concentration and calculated value of the light intensity has been drawn as Fig.6.

IV. CONCLUSIONS

The work focused on the design of one online lab-on-chip continuously monitoring platform system which can be helpful for medical, pharmaceutical and environmental monitoring. And simple Bioluminescent Measurements of ATP had firstly been used which proved to be successful. T conjunction operator combing with the SFA makes the whole system continuous and Y shape internal ribbed micro mixers were used to reach a good mixture effects in a small space. Basic simulations and the measurement had been done, which proved it can be the successful monitoring useful tool.

With the development of luminescent techniques applied to bio analysis [14], it is important to find a simple and reliable method to do the measurements. PMT [9] has been used in this paper; some other methods will also be tried, including polycarbazole photodetector [15] and liquid scintillation counter [16]. And to meet with the aim of multifunctional design, more couples of important toxicants and biosensors will be texted in my further work, especially on the urinary tract infection. I believe this design is the successful connecting between medicine fields to the physical analyzers.

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Detection of Urinary Tract Infections on lab-on-chip device by measuring photons emitted from ATP bioluminescence

Shilun Feng, Tao Dong*- *IEEE Member*, and Zhaochu Yang

***Abstract*— A microfluidic Lab-on-chip (LOC) platform for in vitro detecting Urinary Tract Infections (UTI) for clinical diagnostic applications has been built. Based on one commercial adenosine 5'-triphosphate (ATP) assay kit, one chip designed before was applied to detect UTI with the help of photomultiplier tube (PMT) and quantitative determination was made by measuring the photons of light emitted in the bioluminescent reaction of ATP with the enzyme luciferase. The chip had been tested and materials had been well prepared before testing the PMT detecting system. The data from PMT were visualized by the Labview™, appearing good linearity between voltage values and the concentration of the ATP ranging from 2×10^{-12} M to 2×10^{-8} M. Fresh urine sample with different amounts of *Escherichia coli* had been measured by the system, appearing good linearity trend between the voltage values and number of the *E.coli*. This study successfully expressed the concept of measuring ATP directly in the urine to quickly and accurately detect UTI on a microfluidic chip.**

I. INTRODUCTION

The urinary tract is the most common organ system to experience bacterial infections [1]. Urinary tract infections are the most common infections in both hospitalized and community patients [2], which are common infects causing serious morbidity and significant expenditures in healthcare dollars and lost wages [3].

Use of bioluminescence as a urine screen was first described in 1944 [4]. Application of the luciferase enzyme and luciferin to the detection and quantitation of bacteria was originally described by Chappelle and Levin, who used a luminometer to measure bioluminescence (as relative light units [RLU]) [5]. Firefly lantern bioluminescence is produced by a biochemical reaction in which the luciferase enzyme utilizes ATP in the oxidation of luciferin to adenyl-oxyluciferin, with the concurrent release of photons in proportion to the amount of ATP that is converted to AMP. The mechanism of the bioluminescent reaction occurs [6] as follows:

$$\text{ATP} + \text{luciferin} + O_2 + \text{luciferase} \rightarrow \text{AMP} + \text{oxyluciferin} + CO_2 + \text{PPi} + h\nu \quad (1)$$

Luciferase produces cold light (~560 nm) by the ATP-dependent oxidation of d-luciferin. Consequentially, the amount of ATP is directly associated with the amount of emitted light by luciferase, which in turn represents the amount of cells in the solution.

Although an incubation step is required, urinary ATP concentration luminance analysis method can provide the rapid and reliable result. Several problems including the presence in urine of free nonbacterial ATP, the intracellular ATP contained in somatic cells, and luciferase inhibitory substances and the variation in ATP content among bacterial species appears in initial attempts to apply this methodology to the enumeration of bacteria in clinical urine specimens, all of which compromised the quantitative aspect of the assay [7-8]. These problems were efficiently solved by using buffers and reagents for the release and destruction of nonbacterial ATP in clinical urine specimens. The reported threshold for positivity was 10^5 CFU/ml, with a sensitivity of 86 to 95%, a specificity of 75 to 82%, a positive predictive value of 36 to 77%, and a negative predictive value of 88 to 95% [9-10]. It is reported that urinary ATP concentration analysis is useful for determining urinary tract infection and renal damage caused by drugs and by means of the firefly luciferin-luciferase method, reference value was established as 1.77×10^{-10} M to approximately 7.70×10^{-9} M. [11]

Another paper reported that using the microfluidic device with ATP standard solutions, the bioluminescence intensity was linearly correlated with 2×10^{-12} M to 2×10^{-8} M of ATP [12] , which was more accurate, however, it was not applied for detecting UTI. Thereby, in this paper, I combine the usage of microfluidic device to detect the ATP as assay, it can not only quickly get the accurate result ranging from 2×10^{-12} M to 2×10^{-8} M but also prevent the poor stability and high cost of luciferase and d-luciferin in bioluminescent reactions .

Photomultiplier tube (PMT) based detection module is also installed for quantify weak signals of the bioluminescence reaction (quorum sensing) [13]. It has been instantaneously recording the maximum bioluminescent light signal emitted from the whole reaction process by measuring the changes of the voltages on the Labview™. Usage of PMT by measuring wavelength to count luminance photons one by one had been studied and would be used in the future researches, which has better sensitivity and seems promising. Considering the structure of one chip [14] which is quite suitable for the firefly luciferin-luciferase method and PMT

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T. Dong (IEEE member: 90889664) is with the department of Micro and Nano Systems Technology (IMST, TekMar), Buskerud and Vestfold University College (HBV). (PhD T. Dong is the corresponding author, e-mail: Tao.Dong@hbv.no; phone: +47 3303 7731 fax: +47 3303 11 03).

S.L. Feng is with the department of Micro and Nano Systems Technology (IMST, TekMar), Buskerud and Vestfold University College (HBV), Norway. (E-mail: shilun.feng@student.hbv.no).

Z.C. Yang is with the department of Micro and Nano Systems Technology (IMST, TekMar), Buskerud and Vestfold University College (HBV), Norway. (E-mail: Zhaochu.Yang@hbv.no)

detection, I applied this chip for the application of detecting UTI. The chip is composed of two counter-flow micromixers, a T-junction droplet generator and time delay channels (TD-Cs). Urine sample which had been pretreated and nucleotide releasing buffer were imported into the first micro mixer at rate of 1μL/min where the ATP would be totally released. And then the mixed solution and *Luciferin* were imported into the second micromixer at the rate of 1.8 μL/ min, after which the droplet generator encapsulated them inside aqueous droplets separated by air. Air flow was the disperse medium, which could guarantee sufficient oxygen supply for the cells in droplets and also control the time that solution stayed in the channel of TD-Cs. The TD-Cs is a round spiral channel which can provide the platform for the detection of the PMT. The system showed high reliability and stability through numerical and experimental investigations. In the microfluidic domain, the analyzer is based on continuous flow, using syringe pumps. The system includes the flow in the chip, the PMT photon detecting and electrical circuit to connect to the PC, and the software including Labview™ to visualize the data. Continuously, it had been tested by the ATP standard solution. After which, fresh normal Urine samples had been mixed with different amounts of *E. Coli* cells and tested, which proved feasibility of detecting UTI.

This paper have successfully given out the concept of using microfluidic chip as a platform providing bioluminescent reaction happening and using the PMT system detecting the luminance photons emitted and finally getting the signals for detecting UTI accurately and quickly.

II. MATERIALS AND METHODS

A. Preparation of biological samples

ATP Assay Kit (119107-1KIT) was purchased from Merck Chemicals Ltd. (Padge Road, Beeston, N G9 2JR Nottingham, United Kingdom). The reagent is constituted of one vial of Luciferase ATP Monitoring Enzyme, Enzyme Reconstitution Buffer, a bottle of Nucleotide Releasing Reagent, one vial of ATP Standard, and a user protocol. The assay is based on the firefly luciferase-catalyzed oxidation of D-luciferin in the presence of ATP and oxygen, where the amount of ATP is quantified by the amount of light produced.

Prepare the ATP standard solution by dissolving 1mg ATP into 1ml of H_2O, and store it in the fridge at -20℃, which will be used to detect the whole system. Remove different volumes of normal urine medium and 10^4-10^6 *E.coli* cells cultured in Luria Broth (LB) medium with 10g/L Tryptone, 5g/L Yeast extract and 10g/L NaCl as inlet 1, nucleotide releasing buffer as inlet 2, they are mixed at first Micromixer for about 5min at room temperature. Reconstitute ATP Monitoring Enzyme with 2000 μL of the Enzyme Reconstitution Buffer freeze at -70℃ for future use and then use 1ul ATP monitoring enzyme being diluted with 49ul Nucleodtide releasing buffer as inlet 3 for the chip. ATP and enzyme solutions were pre-treated by Multifunctional sample preparation kit [15] and injected into the microfluidic chip at different flow rates through 3 inlet ports separately.

B. Installation of the microfluidic chip

In this study, a designed chip [14] as shown in Fig. 1,

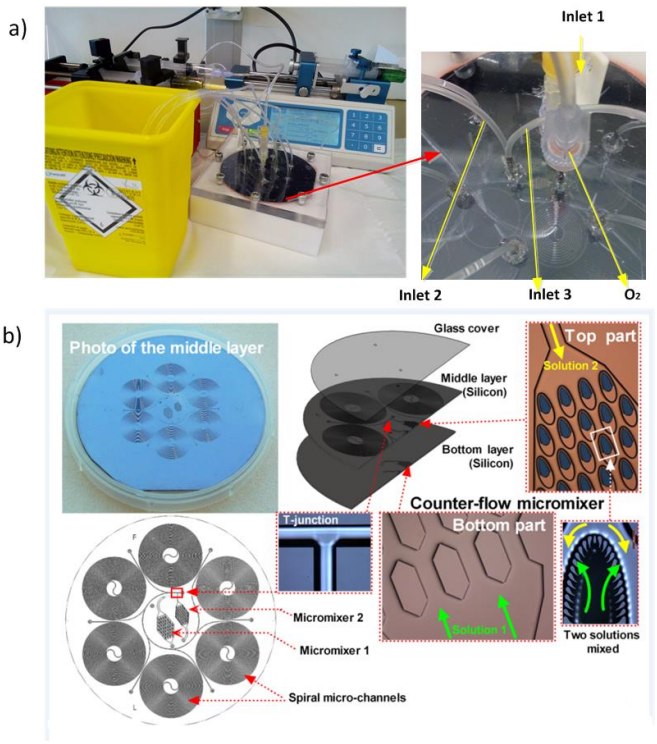

Figure 1 Description of the cell-based LOC device; (a).Application of LOC chip; inlet 1 for the samples, inlet 2 for the nucleotide releasing buffer and inlet 3 for the mixed ATP monitoring enzyme; (b).Construction of the cell-based LOC device [18]; The chip has three layers, but the three major structures are in the middle layer, including two counter-flow micromixers, a T-junction droplet generator and six spiral micro-channels. A photo of the device is shown in the top left corner. The schematic and micrographs of the counter-flow micromixer are illustrated at the top right part, which houses the counter-flow units (on the middle layer) and the inlet channels (on the bottom layer). The tiny inlet port is located in the center of the counter-flow unit. The sample, buffer solution and the cell suspension are mixed and used to form droplet within the air flow.

composed of two micromixers, a T-junction droplet generator and six TD-Cs was used. The two micromixers recreate optimal environmental conditions for the release of the ATP from constant cells while the droplet generator encapsulates them inside aqueous droplets separated by air. The droplet flow had been investigated through numerical simulation since it represents an innovative two-phase flow that allows to oxygenate living cells and to operate at lower pressure than the common two-phase flow. The concentration of ATP in different levels of E. coli for different urine samples can be measured by quantifying the light intensity at the TD-Cs.

C. Building of the detecting system

The whole system is shown in Fig.2, which includes the microfluidic chip, the PMT photon detector and read-out circuit connecting to a PC with the software of Labview to visualize the data. Form the voltage appearing on the Labview which reflects the intensity of the light, getting the relationship of the voltage with the concentration of the ATP. With the decrease of the concentration of the ATP that applied, we can detect the lowest detection concentration. After the whole system being built, different concentration of *E.coli* in the urine sample was transported to the chip system; after going through the chip, the signal was recorded with the PMT detecting system. After that, the signals were visualized by

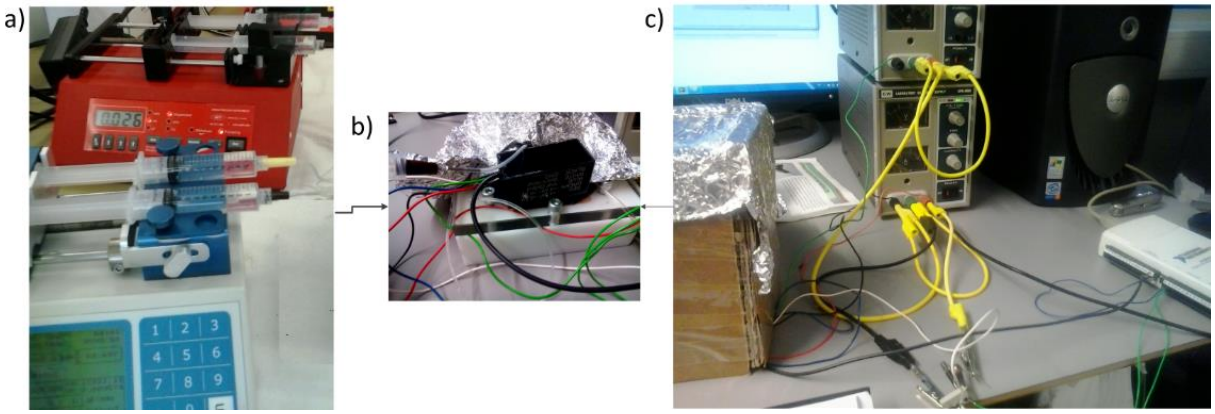

Figure 2 Testing of the whole system; (a).the flow of the chip. (b).the PMT photon detecting; (c). electrical circuit to connect to the PC and the software including Labview™ to visualize the data

Labview on the PC. the data of UTI can easily observed and shared [16].

III. RESULTS AND DISCUSSION

A. *Testing of the whole system*

Software of this system was designed, by measuring the output which is linearly corresponding with signal intensity. Hardware of this system was also designed, using two power supplies, one constant 4.7kΩ resistor, one 1kΩ potential resistor, several wires to connect and DAQ National Instruments. Both the testing of the software and hardware of the PMT and basic measurement of the voltage reflecting the bioluminance light intensity emitted from the ATP standard solution had been done, which proved its application.

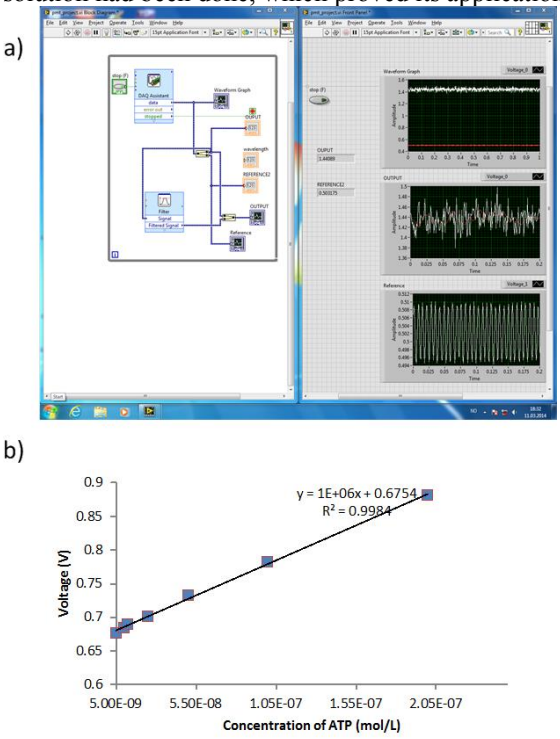

Figure 3 Testing of the whole system; (a) Labview™ was used to visualize the data by measuring the change of the voltage; (b) ATP standard solution was used to calibrate the whole system.

B. *Measurement of UTI*

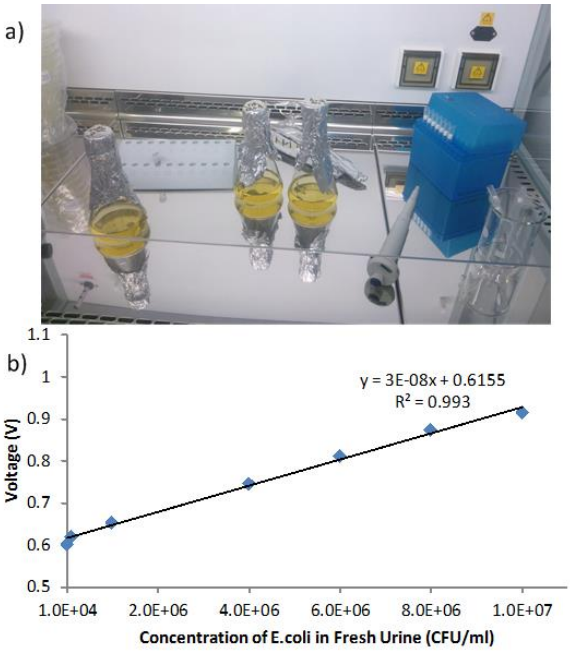

Figure 4 Detecting of the Urine with different amounts of E.coli

Pick up constant number of the *E. coli* growing in Luria Broth (LB) medium with 10g/L Tryptone, 5g/L Yeast extractand 10g/L NaCl and then dilute them in the constant volume of fresh urine and then transport them into the system separately. As Fig. 4 shown, the voltage has the linear trend with the concentration of the *E. coli*, which proves feasibility of detecting the UTI. *E.coli* cells were kept for 8 hours in the fridge at 4℃， however no signals were detected, which prove that the system can only detect the fresh samples.

C. *Testing the mixture efficiency of the microchip*

Several experiments had been done for the microfluidic features of the chip including the refining microfiltration [17] and different inlets rates determine the mixture effect. Finally when urine sample form the inlet1 being at rate of 1μl/min, nucleotide releasing buffer solution flowing at 1μL/min from

inlet 2, and mixed with mixed ATP monitoring enzyme flowing at 1.8μL/min from inlet 3 and mixed solution was capsuled by the Air flow at the rate of 1.5μL/min, which provided a stable flow and meeting with the optimal condition for chemical reaction and detection.

D. The simply and more precise PMT detection system

The aim of this paper is to apply the PMT detecting system to read the photons of the ATP luminance. A lot of investments have been done, however, what the experiments had been down is to have built a system using Labview™ visualized the dates form the luminance accumulated light by measuring the voltage appearing. In the latter work, improvement of PMT detection system will be done, the light signal can be measured by photons one by one, thereby creating the highest sensitivity and other methods will also be tried, including polycarbazole photodetector [18-19].

E. The efficiency of the detecting UTI on lab-on-chip

Combing with the ATP assay and the microfluidic chip is designed to detect the UTI, which has the advantages of both fast reliable detecting and more precisely with very lower limit detections. Using LOC also prevents the poor stability and high cost of *luciferase* and *d-luciferin* in bioluminescent reactions. And it can also reused, which makes the less expenses. Moreover, because of lab-on-chip which is small and portable, more related experiments could be investigated based on this platform and it may render the complicated immuno-NASBA assays convenient to common users without special training [20].

IV. Conclusion

ATP luminance method to detect UTI was applied on the microfluidic chip. One ATP commercial assay kit and one designed microchip had been combined and with the help of the PMT detecting system, the UTI could be quickly and precisely detected by reading the visualized data from the luminance photons emitted from ATP firefly luminance reaction. This combined method is promising because microchip can protect the ATP away from polluting to get an accurate result, moreover, by reading photons, the lower concentration can be figured out without urine culture, which provides the quick detection and saves high cost of *luciferase* and *d-luciferin* in bioluminescent experiments. In this paper, only simple experiments have been done for this promising conception, the more precise experiments will be done in the future.

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Diaper-based UTI test for elder people by measuring nitrite through Ion selective electrochemical sensor

Shilun Feng, Tao Dong *

Department of Micro and Nano Systems Technology (IMST, TekMar), Buskerud and Vestfold University College (HBV), Norway.

*Tao.Dong@hbv.no

Keywords: UTI, elder people, adult diaper, nitrite.

Abstract. The urinary tract infections (UTIs) are common infection diseases in the hospitals, especially for the elder people. Detecting UTI for the elder people is difficult and time-consuming procedure. Since measuring nitrite directly in urine to detect UTI is promising, commercial electrochemical sensor had been assembled and the ability to detect the UTI on the diaper had been investigated. With one commercial electrochemical sensor for nitrite in water, one system was built to quickly and precisely detect different concentrations of nitrite in the fresh urine. The experiment had shown the good result that the nitrite can be detected in the fresh urine by the electrochemical sensor with the relations as E=224.7-21.259lg (c+13.476), where E is the potential voltage and c is the concentration of the additive nitrite solution. In this paper, diaper also had been dealt with to extract out the urine and more sampling modules had been investigated.

Introduction

UTIs are among the most prevalent infectious diseases in the general population [1]. Moreover, UTI is a common reason for hospital admission in patients aged 75 and older [2]. It is challenging for frail elderly people to detect the cases of symptomatic UTI with an over diagnose tendency [3].

Early diagnosis of UTIs was mainly by urinalysis reagent strip and/or microscopy which can also determine the performance of urine cultures. The objective of this work was to evaluate the sensitivity, specificity and sensitivity of urine [4]. In a meta-analysis of studies performing urine dipsticks on older patients between 1990 and 1999, the sensitivity and specificity were calculated as 82% and 71%, respectively, for nitrites or LE positivity [5]. However, a study has suggested that urine dipsticks given the high prevalence of ASB and pyuria, the percentage of suspected UTI that could be excluded was small (those tested accounting for 12%, all suspected UTI accounting for 7%) [6]. Even urine dipstick test has a very poor sensitivity when performed on randomly selected urine specimens [7].Further difficulties with this method include obtaining urine samples (especially in patients with incontinence or cognitive impairment) and sample contamination. Similarly, the high prevalence of ASB means that urine culture results alone cannot be used to diagnose clinically relevant UTI in elderly people. Moreover, the high prevalence of bacteriuria in elderly individuals makes it difficult to know if a new symptom is related to bacteria in the urine. There are different views concerning this relationship and bacteriuria often leads to antibiotic treatments [8].

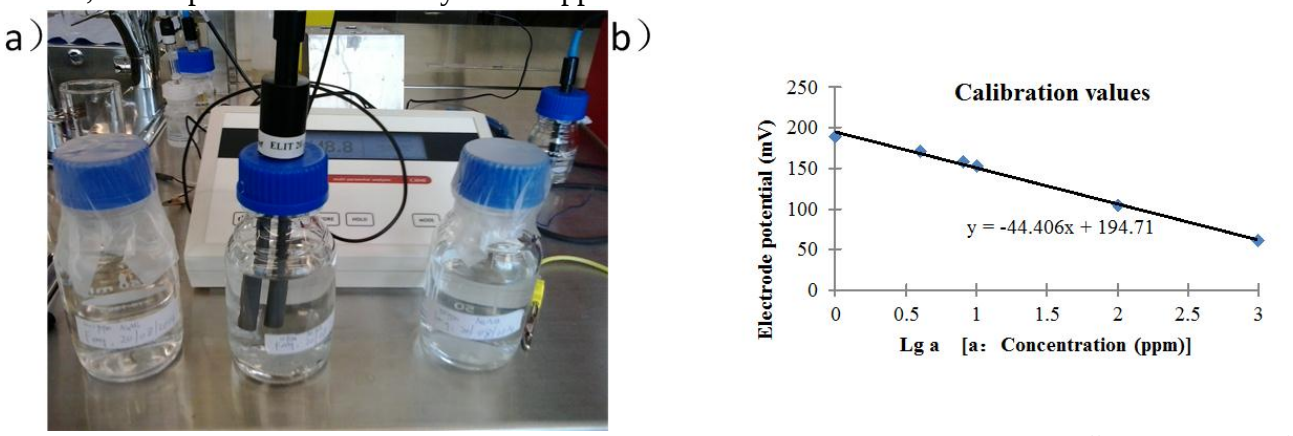
Diapers are often employed in UTI patients, so the diaper might become a sampling tool for urine. To the elderly patients, collecting enough urine for UTI tests usually turns into a painful and time-consuming procedure. The diaper-based UTI test will be a convenient solution, but nowadays few diagnosis tools are designed to test the urine samples on diapers. To develop a point-of-care real-time ‘on diaper’ detector for nursing staffs to easily distinguish UTI patients will be promising.

Urinary nitrite has been used as an indicator of urinary tract infections for many years [9].Nitrate is reduced to nitrite by the enzyme nitrate reductase, which is present in gram-negative bacilli causing urinary tract infection. If we know the concentration of nitrite quantitatively, then we know the possibilities of the UTI. It has been suggested that testing be performed only on urine that has remained in the bladder overnight or for at least 4 h to allow for a higher concentration of urinary

nitrite [10]. Therefore, dealing with the used diaper in 4h of the elderly people on the nitrite quantitatively detecting could be promising and of great importance.

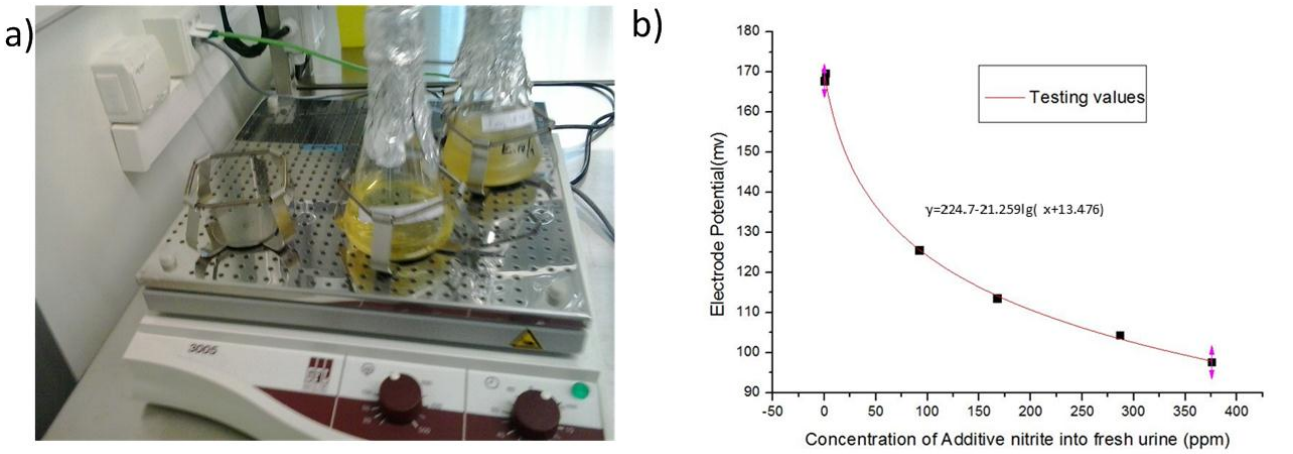
Experimental section

Assemble and calibrate electrochemical sensor. Different concentration of Nitrite solution with 1000ppm, 100ppm, 10pm, 1pm, 0.1 ppm and 0.01ppm were used to calibrate the electrochemical sensor, which proves the feasibility for its application to detect the different concentration of nitrite.



Picture 1 Assemble and calibrate the electrochemical sensor. a) Assemble the commercial electrochemical sensor; b) using different concentration of Nitrite solution to calibration the electrochemical sensor .

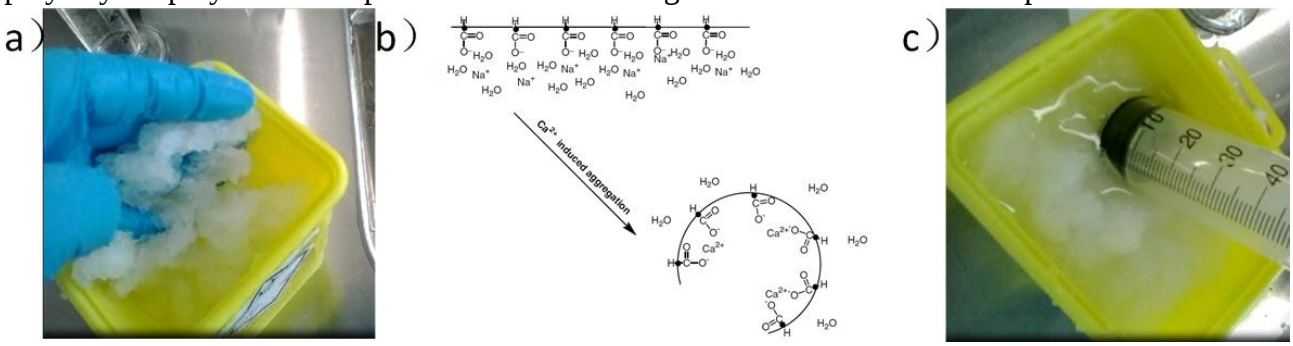
Detect the UTI. Fresh urine was mixed with different volume $NaNO_2^-$ and prepared [12, 13].



Picture 2 Tests of Electrochemical sensor. a) Preparation of the urine samples with different constant concentration of nitrite; b) using different concentration of Nitrite solution to mix with the fresh urine to test the electrochemical sensor.

They were detected and measured, the results shows that the electrochemical sensor has the ability to detect the amounts of nitrite concentration in the urine. If there is one database for the nitrite of UTI, there will be a promising marker for detecting UTI precisely.

Extract urine from the disposable diaper. Two popular brands of diapers that contain polyacrylate polymers were purchased from a local grocer and tested. The absorption of the

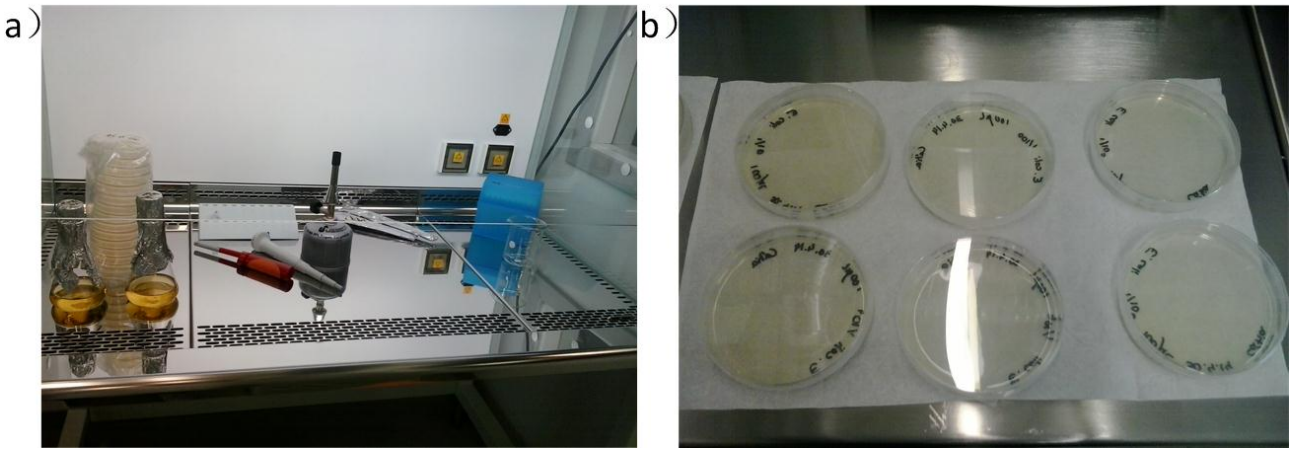


Picture 3 Urine Extraction from disposable diaper. a) shape of polyacrylate absorbent Powder of one whole diaper after absorbing 500ml DI water; b) principle of the absorption for polyacrylate absorbent Powder(The Ca2+ ions can bind the carboxylate groups of the polymer and neutralizes the

polyanions. Due to the hydrophobic nature of the backbone, the polyacrylate chain collapses.)[11] c) water release out after adding 100ml 150 g/l of calcium chloride solution.

polyacrylate polymers inside one diaper had been proved, which could absorb 500ml water without one droplet leaking out. Based on one principal of putting Calcium salt solution can make the poluacrylate chain collapse and leak out the urine [11], 100ml 150 g/l of calcium chloride solution was added and finally get 480ml salt solution by Syringe, which provides solution for further works.

Cell culture E.coli. E.coli was cultured to the constant amount for the investigation of mechanism on E.coli converting nitrate to nitrite.



Picture 4 Cell culture E.coli. a) details for the cell culture; b) accounting the numbers.

Further works

More precise works will do including inventing integrated electrochemical sensor which can not only extract urine form the used diaper directly without contaminating the samples but also portable; proving nitrite can be the solely and reliable marker to detect UTI; giving out the design that can quickly and accurately detect the UTI on diaper and send to the data to the nurse [14]. Constant numbers of E.coli will be put into the fresh urine respectively and measure the values with the time changing in 4h, thereby proving the mechanism that E.coli can convert nitrate to nitrite. Different numbers of E.coli with 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 will be mixed with fresh urine for 1h and test for the provident that different E.coli can provide different amounts of E.coli and thereby adding the possibility that measuring nitrite can detect the UTI. Electrochemical sensor can be successuffly read and acquire the needed data follows the well based biosensor technology [15, 16].

Conclusion

Methods of extracting urine form the diaper for the elder person had been investigated. Urine can be extracted by the syringe after adding the calcium salt solution; however more sampling module will be designed and fabricated. Electrochemical sensor had been successfully assembled and tested. Different numbers of nitrite with fresh urine had been tested by the electrochemical sensor, which proves its feasibility for detecting UTI. E.coli had been cultured for more precise works to be done. The conception of using electrochemical sensor to detect the nitrite of urine from the diaper of the elder people proves reliable and very promising.

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Quantitative detection of *Escherichia coli* and measurement of urinary tract infection diagnosis possibility by use of a portable, handheld sensor

Shilun Feng, Lars Eric Roseng, Tao Dong*
Department of Micro and Nano Systems Technology (IMST, TekMar), Buskerud and Vestfold University College (HBV), Norway
*Author to whom correspondence should be addressed; E-Mail: Tao.Dong@hbv.no; Tel.: +47 3100 9321; Fax: +47 3303 1103.

***Abstract*—Electrochemical nitrite sensor was used to quantitatively detect the nitrite concentration in urine and by building the approximate relationship between the nitrite concentration and number of *E.coli* bacterias, electrochemical nitrite sensor can count the numbers of Escherichia coli and do the Urinary Tract Infection (UTI) Diagnosis. Electrochemical nitrite sensor was assembled and calibrated, the artificial urine sample was detected; the feasibility of electrochemical nitrite sensor including the errors effect had been checked and proved at around -5.1~2.3%; the possibility to detect artificial UTI urine sample out is around 95.5%; the approximate relationship between the number of *E.coli* and electrode potential had been built as E=228.3193-3.78225×Ln (N+2.29101e6) , thereby building the relationship between UTI possibilities and the measurement. Finally, the conception and design of electrochemical sensor array had been made, thus to measure different biomarkers for the maximum possibilities of UTI and can show the data of the possibility of UTI directly on the screen. Furthermore, it can easily be used and transported for the home-users or patients in hospitals.**

***Keywords*—Urinary Tract Infection; Nitrite; Escherichia coli bacteria; Electrochemical sensor**

I. INTRODUCTION

Urinary tract infections (UTIs) are among the most common bacterial infections in humans, the majority of bacterial strains including *E.coli* which is the governing causative species[1], *Klebsiella*, *Proteus*, *enterobacter* causing UTI have a nitrate-reducing capacity to produce the nitrite. Nitrite is known to give a sensitivity on 35% to 85%, but highly specific [2].

Since the 1920s, when the urinary nitrite test was first developed, the dipstick assay has been an important component of modern UTI diagnosis. The growth of bacteria in slightly acidic urine is inhibited if exogenous nitrite is added [3, 4]. This inhibition was potentiated in the presence of the reducing agent ascorbic acid. Indeed, acidification of urine has been used in traditional medicine for the prevention and treatment of urinary tract infections [5], although there are not enough convincing findings for this concept from clinical trials. In this paper, we have expanded the role of nitrite beyond diagnosis of infection and applied in the new electrochemical urinalysis system. The urinalysis system for home users arouses general interest of researchers, since urine tests are also suitable for household daily monitoring of personal health conditions. The strips are too simple to fulfil the demands of home users. The users are required to handle their urine samples and the commercial strips step by step; moreover, the phenomenon on the strips has to be evaluated by home users themselves according to the user manual and it really change with the time going. Many home users are confused by the strip tests.

In this article, a new-concept automatic urinalysis system using electrochemical sensor by detecting nitrite quantitatively proposed to provide personal urinalysis services for home-users were pointed out. The system will involve multiple functions of automatic sampling [6], rapid detecting, auto-decontaminating [7, 8]. Moreover, it can analyze and show the data of the possibility of UTI directly on the screen and easily used and transported for the home-users by using personalized health information technology (HIT) supporting which is one of the few urinalysis systems with a built-in connection to IT technology and represents that the abundant medical information in urine samples could be measured and exported directly into a web-based medical database [9], thus providing a low-cost solution of automatic urinalysis services for patients in hospitals and home-users.

II. SENSOR PRINCIPLE

Generally, membrane-based biosensor can be separated into two parts: Potentiometric and Voltammetry methods. In this paper, Ion selective sensor which belongs to potentiometric had been used which has three main basic components: a) reference electrode: gives reference for potential measurement; b) indicator electrode: where species of interest is measured; c) potential measuring device, as *Fig. 1*.a) shows and it is based on measurements of the potential of electrochemical cells in the absence of appreciable currents as *Fig. 1*.b) shows. In the application as in Nernst equation [10]

$$E = E0 + (2.303RT/nF) \times Log\ (a) \quad (1)$$

Where E = the total potential (in mV) developed between the sensing and reference electrodes. E0 is a constant which is characteristic of the particular ISE/reference pair. (It is the sum of all the liquid junction potentials in the electrochemical cell); 2.303 is the conversion factor from natural to base10

logarithm; R is the Gas Constant (8.314 joules/degree/mole); T is the Absolute Temperature; n is the charge on the ion (with

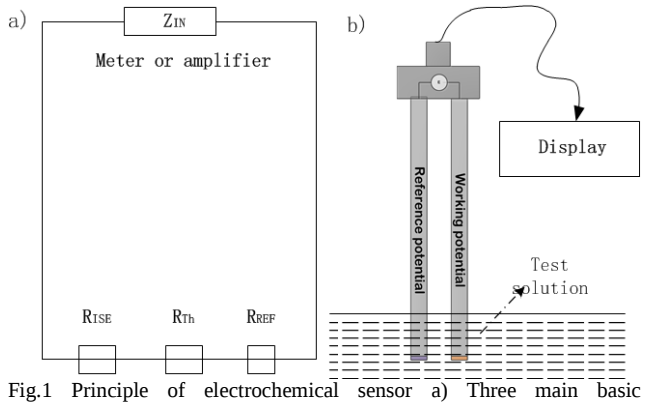

Fig.1 Principle of electrochemical sensor a) Three main basic components; b) detect process.

sign); F is the Faraday Constant (96,500 coulombs per mole); Log(a) is the logarithm of the activity of the measured ion.

III. RESULTS & DISCUSSION

A. *Calibrate the electrochemical nitrite sensor*

Assemble and calibrate of Commercial Nitrite Ion-Selective Electrode sensor (nico 2000: ELIT 8071) had been done and a calibration curve had been drawn as

$$E=-45.347\log a+195.21 \quad (2)$$

Where a is the concentration of nitrte, and E is the potential of the sensor. Electrode is also used for the testing of nitrite ions ($NO2^-$) in fresh urine. Quantitative detection curve of sensor had been done to prove its feasibility to detect nitrite in urine solution, with the fitted relations as in

$$E=224.7-48.95\times Log\,(a+13.476) \quad (3)$$

Where E is the electrode potential and a is the concentration of nitrite ions. The characteristic of commercial device is: length of body excl gold contact is 130 mm; length of body incl gold contact is 140 mm; diameter of body is 8 mm; DC resistance at 25°C is smaller than 2.5 MOhm and minimum feasible sample volume is 5 ml. Preconditioning time is at least 5 minutes; optimal pH range is between pH 4.5 to pH 8; temperature range is 0 to 50℃. Recommended concentration range is 0.5 to 500ppm and response time is smaller than 10 seconds and potential drift (in 1000 ppm) is smaller than 3 mV/ day (8 hours).

Different urine samples were used to test the reliability; the results show slightly differences around the value 13.476ppm. There calibration works both in standard nitrite solution and urine had been done in [11].

B. *Feasibility of the detection of UTI*

Temperature brings error to the detection. Many samples were detected with different controlled conditions, which gets that errors of this electrochemical sensor keeps the range at -

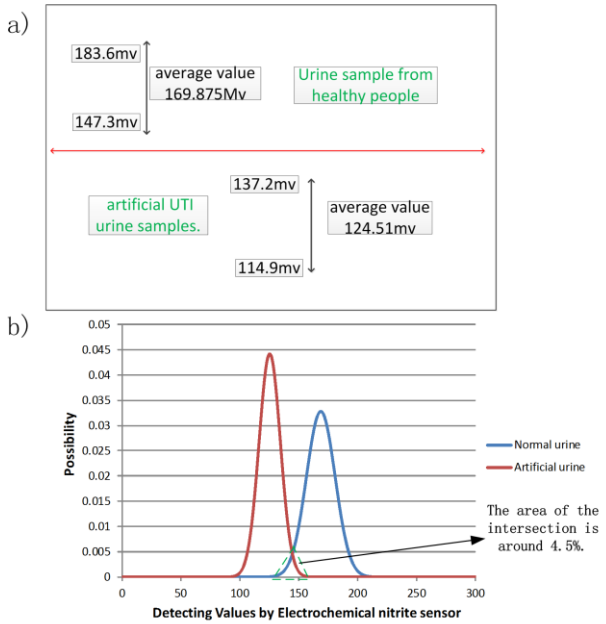

Fig. 2 Detect the possibilities of electrochemical nitrite sensor for the UTI. a) The values detected from normal urine and UTI urine; b) the possibilities of the intersection between two groups of detection

5.1~2.3%. 20 normal urine samples from healthy people and 20 artificial urine samples by adding nitrite had been tested to prove the feasibility of the electrochemical sensor. The results are: for the health urine samples, ranging from 147.3mV to 183.6mV, with the average value 169.875mV; for the artificial UTI urine samples, ranging from 114.9mV to 137.2mV, with the average value 124.51mV, as *Fig. 2* a).

Standard distribution was drawn based on the detection values as *Fig.2*b). The intersection was calculated with the value around 4.5% as *Fig.2*b), which means this electrochemical sensor can differentiate the artificial urine samples from the normal persons' samples by the possibilities at around 95.5%. $\alpha + \beta = \chi.$ (1) (1)

C. *Effects of nitrite on bacteria growth*

Urine is an excellent culture medium for bacteria that causes urinary tract infections. Indeed, at a basal urinary pH and even in slightly acidic urine the bacterial growth was very good [12]. In contrast, the growths of *E.coli*, *P.aeruginosa* and *S.saprophyticus* were dose-dependently inhibited by nitrite in acidified urine. The inhibitory effect of nitrite was greater at lower ph. Addition of ascorbic acid (10 mM) further enhanced the inhibition of bacterial growth by nitrite. Increasing concentrations of nitrite(0-500 μ M) with a fixed concentration of ascorbic acid (10mM) caused a dose-dependent inhibition of *E.coli* growth. And the higher the nitrite concentration, the stronger inhibition will be. However if bacteria were pre-incubated in basal urine without addition of sodium nitrite, no inhibition was noted when the culture was transferred to the acidic urine. This indicates that a normal diet in a patient treated with urinary acidification is probably insufficient to excrete enough nitrates in the urine to achieve antibacterial effects. Experiments of above experiments were referered

from [13] and done in the lab. Moreover, nitrate was found to be stable in urine and plasma samples when they were incubated for 2h at 37℃ or stored for 24h at 4℃. [14]

D. *Approach for detecting Kinetics mechanism of converting nitrate from normal diet into nitrite by E.coli*

Prepare the *E.coli* (ATCC25922) cells for 1.85×10^9 CFU/ml; dilute *E.coli to* 1.85×10^8 CFU/ml, 1.85×10^7 CFU/ml , 1.85×10^6 CFU/ml, 1.85×10^5 CFU/ml and 1.85×10^4 CFU/ml with enough human urine of a health volunteer at 37℃ at the ration 1: 100 (the volume of the culture medium to the volume of urine) respectively. Nitrite was measured by the electrochemical sensor after 24h. The results showed different numbers of *E.coli* generated different concentrations of nitrite. And then relationship between the numbers of *E.coli* and electrode potential can be found as *Fig. 3*a).

$$E=228.3193-3.78225\times Ln\ (N+2.29101e6) \quad (4)$$

Where N is the numbers of *E.coli*, and E is the potential of the sensor. It has the similar equation as the Equation (3). The relationship is only the approximate link since there exists uncertainty of equation (4) because of different resources of human urines, different growth rate of *E.coli* bacteria in different environment, and different measuring errors of the sensor. However, we can get the maximum/rms difference of real samples used to obtain eqn (4) with respect to the obtained curve, which could help to further set the detection possibility. Continuously, concentration can be calculated out by Equation (3) after giving out the detection values. Thereby, this test also gives the relationship between the number of *E.coli* and concentration of nitrite in the urine sample, which is almost linear relationship. Finally, the relationship between UTI possibilities and nitrite detection can be made. Moreover, if more date and experiments had been done, we can form the database for the relationship between the UTI possibility and the detection values, thereby screening UTI quickly and quantitatively detecting nitrite using electrochemical sensor.

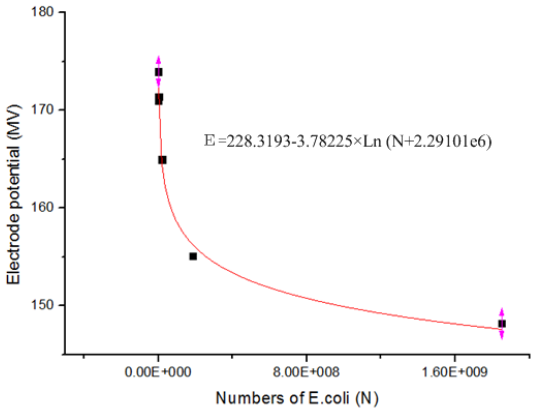

Fig. 3 Approach for detecting Kinetics mechanism of converting nitrate from normal diet into nitrite by *E.coli* a) Relationship between the numbers of *E.coli* and the measured electrode potential; b) verified by the standard normal urine strips.

Used solutions were tested by standard strips. We found higher numbers of *E.coli*, *the pinker the nitrite pads are*, *which means* producing higher concentration of nitrite， as *Fig.* 3b) shows.

IV. CONCEPT DISCUSSION & FUTURE WORK

A. *Nitrite detection possibility for the UTI detection*

Relationship between number of *E.coli* and nitrite concentration had been investigated and can get the approximate relationship, however, more specialized experiments and relationships should be found to form the database, and with the results got, since exact high enough *E.coli* number(normally bigger than 10^5 CFU/ ml) in the urine stands for the getting UTI infection, it is so easy for us to do the UTI diagnosis quantitatively without cell culture. There are still some undetermined aspects in the detection including the nitrate in human beings food; the time for the *E.coli* to converting the nitrate; the pH of the urine which would determine the process of the converting; the other bacteria which can generate the nitrite and the temperature and of course the noise for the electrochemical sensor detection, all of which give the errors for the detection in some extents. However, as one promising monitoring tool which can be used for home-users, we can use this kind of detection method to get the detection possibility of UTI infection quickly and conveniently.

B. *Electrochemical sensor array for the UTI detection*

From [11], the feasibility of the sensor to detect UTI out has been proved. From *Fig. 3*, we tried to build a relationship between the nitrite concentration and the numbers of *E.coli*, Electrochemical sensor array can be realized for rapid multiplexed detection of pathogens [15]. Multi-sensing and data fusion are necessary. If we use the electrochemical sensor to detect the nitrite, we can detect the UTI at some possibility. And the signal can be frequency dominated, which can be described as F(c, f1). Similarly, we can also detect other biomarkers (for example, alcohol, Leukocyte enzyme, Urinary Lactoferrin) to improve the possibilities for the UTI as the signal function F(c, fx) and show on the screen respectively. If we can measure the nitrite and LE or LF quantitatively at the same time, we can measure the UTI out at the max possibility;

it has the same idea of one paper showing the detection of waterborne pathogens and design and validate them [16]. More work will be done to build and fabricate the sensor array, which can read several biomarkers and do Integral automatic sampling as *Fig. 4*a) shows, rapid detecting, auto-decontaminating and personalized health information technology (HIT) supporting, thus to provide a low-cost

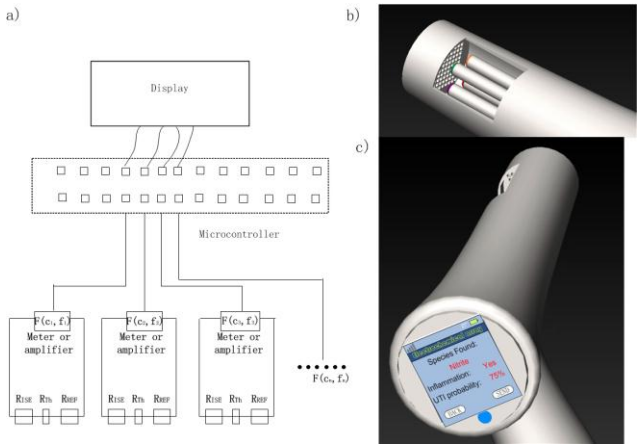

Fig. 4 Design of electrochemical array. a) diagram of the electrochemical array; b) shape of electrode arrays and the sampling structure; c) the screen of the electrochemical array.

solution of automatic urinalysis services for both patients in hospitals and home-users. Moreover, it can analyze and show the data of the possibility of UTI directly on the screen and be easily used and transported.

V. CONCLUSION AND FUTURE WORK

In this paper, commercial electrochemical sensor was assembled and calibrated to detect the nitrite in the urine sample. After building the relationship between the nitrite concentration and the number of *E.coli* bacteria, the electrochemical sensor was used to count the *E.coli* bacteria, which directly build the relationship between the sensor to the UTI possibilities. Finally, in the discussion part, the conception of electrochemical array was pointed out which can not only detect different biomarkers to make the maximum detection possibilities but also combine with the functions such as automatic sampling, rapid detecting, auto-decontaminating and personalized health information technology (HIT) supporting.

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