SALIVA AND BREATH BASED BIOSENSING SYSTEM FOR DISEASE DETECTION AND MONITORING

A Dissertation Presented

By

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to

The Department of Civil and Environmental Engineering

in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

in the field of

Interdisciplinary Engineering

Northeastern University

Boston, Massachusetts

April 2016
This thesis is dedicated to my parents, family, and friends who taught me nothing is impossible, be patient, be positive, be caring and be happy!
ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere gratitude to those individuals who have assisted me during my Ph.D. study and research; I could not have completed the writing of this dissertation without their generous contributions and continuous support.

First, I want to give my huge thanks to my advisor, Professor Ming L. Wang, for his motivation and guidance in all aspects of my work. His in-depth knowledge and innovative vision, enthusiasm, and persistence, have been a great inspiration to me. Through my work with Professor Wang, I have learned that my goal in life is to identify some of society’s greatest needs, and to explore various approaches to resolving them.

I would like to thank the rest of my dissertation committee. I am grateful to Professor Steven W. Cranford, who has taught me so much on molecular dynamics simulation; Professor Edgar D. Goluch, who shared his insights on electrochemical sensing; Professor Veronica Godoy-Carter, who instructed me in molecular biology and enzymatic activities; and Professor Philip Larese-Casanova, who shared with me his knowledge and ideas on environmental chemistry.

My sincere thanks also goes to my senior colleague Dr. Yu Liu, now working at Applied Materials, for training me on microfabrication and sharing her knowledge and experience on gas sensing; and Dr. Yi Zhang, now working at Harman International Industries, for helping us to build electrochemical sensing circuits.

My thanks goes to my colleagues and friends, especially Yunqing Du, Sheyda Nazarian, Yifeng Lu, Yubo Zhao, Zhenyun Qian, Tian Liu, Yuan Gao, Anup Singh, Xin Wang, Hanchul Cho, and Anthony De Ritis, for sharing their expertise with me, and providing undying support and help.

I would also like to express my appreciation to all the staff at the Kostas Nanoscale Technology and Manufacturing Research Center, especially David McKee, Scott McNamara, and Sivasubramanian Somu, for their help with the facilities and experimental issues.

Finally, I reserve my greatest thanks to my family for their endless love, encouragement, and support.
ABSTRACT

The goal of my Ph.D. study is to develop an integrated saliva and breath biosensing system based on nanotechnologies, in order to clinically monitor a person’s health status, disease progress, and treatment results. This system would be noninvasive, allowing for the easy collection of samples without pain or fear, provide rapid and accurate readings, and portable. It would be the first line of defense to treat diseases at home, in a doctor’s office, or at medical clinics.

Our research group has developed a unique and disposable saliva biosensor through a layer-by-layer (LBL) assembly of single-walled carbon nanotubes (SWNT), and multilayer films composed of chitosan (CS), gold nanoparticles (GNp), and glucose oxidase (GOx) to provide accurate, low cost, and continuous glucose monitoring through saliva. Compared to a UV spectrophotometer, our sensor demonstrated excellent clinical accuracy. This disposable noninvasive real-time salivary glucose tracking will prove to be an alternative to blood glucose testing, which requires painful finger pricking.

Furthermore, we have developed a wireless sensor array based on single-stranded DNA (ssDNA) functionalized SWNT to detect some physiological biomarkers in breath. We have successfully detected trace amounts of methanol, benzene, acetonitrile, dimethyl sulfide, hydrogen sulfide and acetone, which are indicators of heavy smoking, excessive drinking, and diseases such as lung cancer and diabetes. Molecular dynamics simulation was used to study the interactions between different DNA sequences with targeted molecules, in order to select optimal DNA sequences for the detection of specific disease biomarkers. A DNA sensor array, built with selected sequences that differentiate many disease biomarkers, can be used in disease diagnosis and monitoring.

The complementary nature of saliva and breath sensing technologies will reduce the number of biomarkers that are required to detect a disease at early stages, rendering their results more accurate and reliable.
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CHAPTER 1

INTRODUCTION

It is of great value to be able to monitor individuals’ health conditions in a noninvasive way. We developed a saliva and a breath sensors based on electrochemical principle and nanotechnologies which can detect disease biomarkers from saliva and from breath respectively. The two sensor systems can be integrated to clinically monitor a person’s health status, disease progress, and treatment results. They are both noninvasive, easy to collect the sample without pain or fear, fast in response, and portable. Both systems were developed based on carbon nanotubes (CNTs) which exhibit remarkable mechanical strength, unique electrical properties and efficient thermal conductivity. Coupled with their high surface to volume ratio, miniature size and ability to be incorporated into electronic devices, CNTs have been widely used in various applications including electronics, optics and bio/chemical sensing. In this work, I will mainly focus on the sensing applications of CNTs, especially on SWNT based chemical sensors and biosensors for individual’s health monitoring. A brief introduction of CNTs is presented first in this chapter.
together with the various types of CNT sensors. A major drawback of SWNTs in gas sensing or biosensing applications is the lack of selectivity due to nonspecific binding. Biomolecules, such as DNA and enzymes, metal nanoparticles, and polymers can be utilized to enhance the specificity of SWNTs to certain molecules. Historical background of breath and saliva analysis will also be introduced in this chapter. Then the motivation and scope of this thesis is presented.

1.1 BRIEF INTRODUCTION TO CARBON NANOTUBES

Carbon nanotubes (CNT) were first produced “accidentally” using an arc-discharge evaporation method and discovered using high resolution transmission electron microscopy by Sumio Iijima in 1991 (Fig. 1.1) [1]. The electron microscopy revealed that each needle-like tube comprised coaxial tubes of graphitic sheets, ranging in number from 2 up to about 50 which was later known as multi-walled carbon nanotubes. The structure of nanotubes originated from graphite where the carbon atoms sit in hexagonal patterns and form flat two dimensional sheets. A single-walled carbon nanotube (SWNT), which can be viewed as a seamless cylinder rolled up from a piece of graphene (Fig. 1.1c), is a hollow cylindrical structure of carbon atoms with a diameter that ranges from about 0.5 nm to 5 nm and lengths of the order of micrometers to centimeters. A multi-walled carbon nanotube (MWNT) is similar in the structure to the SWNT but has multiple nested or concentric walls with the spacing between walls comparable to the interlayer spacing in graphite, approximately 0.34 nm.
Fig. 1.1. Transmission electron microscopy (TEM) images of a) MWNT [1]; b) SWNT [2]; and c) schematic graphs of a graphite lattice, SWNT and MWNT [3].

As illustrated in Fig. 1.2b, SWNT is formed by rolling a graphene sheet into a cylinder along a lattice vector (m, n) in the graphene plane [4]. The values of m and n determine the diameter and chirality of SWNT. The chiral angle $\theta = \tan^{-1}\left[\sqrt{3(n/(2m + n))}\right]$ classifies SWNT into three types: armchair (n = m, $\theta = 30^\circ$), zig-zag (m = 0, n > 0, $\theta = 0^\circ$), and chiral (0 < |m| < n, 0 < $\theta$ < 30°) (Fig. 1.2).

SWNT can be metallic, semiconducting and semi-metallic depending on two structural parameters: chirality and diameter. Armchair carbon nanotubes are metallic (a degenerate semi-metal with zero band gap). Zig-zag and chiral nanotubes can be semi-metallic if the band gap is finite (n - m = 3N, N is an integer) or semiconducting in all the other cases. For semiconducting nanotubes with the same chirality, the band gap is inversely proportional to the diameter permitting each SWNT with distinct electrical properties. SWNTs produced by Chemical Vapor Deposition
(CVD) on supported catalyst are basically semiconducting in nature with roughly 2/3 of them being expected to be semiconducting and the rest being metallic [5].

Fig. 1.2. Relation between the hexagonal carbon lattice and the chirality of CNTs. a) Atomically resolved STM images of individual SWNT: tubes no. 10, 11 and 1 are chiral, whereas tubes no. 7 and 8 have a zigzag and armchair structure, respectively [6]; b) schematic illustration of hexagonal structure of graphene layer; c) SWNT is folded by the sheet along lattice directions: armchair folded in (8, 8), zigzag folded in (8, 0), and chiral folded in (10, -2) [4].

With remarkable electronic and mechanical properties as well as thermal stability inherent from their structures, CNTs have been recognized as one of the best electrical and thermal conductors. Furthermore, its one-dimensional nature with all carbon atoms exposed on the surface makes it a natural candidate for sensor applications. The electric transport in nanotubes is extremely sensitive to local electrostatic environment due to their small size, large surface to volume ratio and high mobility, enabling CNTs to be an idea key element in biological sensors.
SWNT has the largest surface area to volume ratio of any carbon material, 3000 m²/g, as all their atoms are on the surface [7]. SWNTs are made of graphene. Graphene has high intrinsic electron mobility due to its lack of lattice defects as compared to most other semiconductor materials and its unique linear dispersion relation. The diameters of SWNTs are so small that the electron wave vectors along the circumferences are quantized. SWNTs have remarkable electrical performance. High-quality SWNTs can have mobility larger than 10,000 cm²V⁻¹S⁻¹ and mean free path longer than 1 µm. Besides extraordinary conductance, these tiny SWNTs, when made into FETs, can have minimum capacitance coupling with gates. SWNTs are also chemically inert, especially when no defects are present, making them chemically stable and biologically compatible. Because of its molecular-scale size and extreme sensitivity to environments, SWNT is an ideal kind of material to make detectors that are capable to reach single-molecule level sensitivity.

1.2 FUNCTIONALIZATION OF CARBON NANOTUBES

Carbon nanotubes have been widely used for decades as one-dimensional nanomaterials in various types of electronics, optoelectronics and sensor systems. For example, chemical sensors [8-10] (gas sensor [11-12] and biosensor [13]), field emission materials [14], electronic devices [15] and actuators [16]. Particularly, SWNTs are more widely utilized due to their specific electrical, mechanical, optical, chemical, and thermal properties. Diverse applications of SWNTs have been explored, consisting of chemical/biological sensors [17], electrical interconnects [18], thermal heat sinks [19-20], agents for drug delivery [21-22], low voltage, cold-cathode field-emission display [23] and nanoscale circuits for beyond silicon based complementary metal-oxide-semiconductor (CMOS) electronics [24-26]. The electronic properties of SWNTs are one of their most important features, which permit extensive applications in nanoelectronics and sensors [27-28]. The electronic properties of carbon nanotubes can undergo dramatic changes in the presence of trace amount of gases, mechanical deformations or variations in certain operating conditions such as
temperature and pressure. The electronic properties of CNTs, together with their optical, mechanical, chemical and thermal properties, have enabled carbon nanotubes to be one novel material for diverse sensing applications. Changes caused by external stimuli can easily be electrically evaluated in resistor, transistor, or capacitor [28-30]. Thus, it is possible to exploit CNTs as sensing sites for various molecules of interest, from toxic chemical vapors to biomolecules [31-33].

However, a major disadvantage of CNT sensors is the lack of specificity. To solve this problem, an effective method to functionalize CNT sensors is necessary which can enable CNTs to specifically respond to a wide spectrum of analytes. Modification of CNTs with polymers [34-36] and biomolecular complexes [37-39] has shown great enhancement in the specificity and sensitivity of the CNT-based sensors. There is a broad range of novel application perspectives achievable by functionalized CNTs (Table 1.1), however, we focus on the biochemical sensing applications using modified CNTs.

Table 1.1. Potential applications of functionalized CNTs.

<table>
<thead>
<tr>
<th>Applications</th>
<th>Advantages of functionalization of CNTs</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Nanoelectronics</td>
<td>Local modification of the electronic band structure</td>
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<tr>
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<td>[41-43]</td>
</tr>
<tr>
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<tr>
<td>Biochemical sensors</td>
<td>Selective recognition of analyte molecules</td>
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</tr>
<tr>
<td>Field emission</td>
<td>Reduction of the work function for electrons at the tube ends</td>
<td>[49-50]</td>
</tr>
<tr>
<td>Tissue engineering</td>
<td>Improved tracking of cells, sensing of microenvironments, delivering transfection agents, and scaffolding for incorporating with the host's body</td>
<td>[51-53]</td>
</tr>
<tr>
<td>Drug design and delivery</td>
<td>Providing multipurpose innovative carriers, novel nanoscale constructs for drug development</td>
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</tr>
<tr>
<td>Medical technology</td>
<td>Use for disease diagnosis, treatment, new medical composites and scaffolds for regenerative medicine</td>
<td>[55-56]</td>
</tr>
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1.2.1 FUNCTIONALIZATION OF SWNT WITH DNA
DNA is the genetic material of all living organisms. DNA consists two long polynucleotide chains which run in the opposite directions and are twisted around each other right-handedly (Fig. 1.3a) [57]. Each strand of the double helix is a linear chain with a backbone made of sugars and phosphate groups joined by ester bonds. Attached to each sugar is one of the four types of bases, including the purines: adenine (A) and guanine (G), and the pyrimidines: cytosine (C) and thymine (T). The rules of base pairing are: A with T and C with G (Fig. 1.3b). DNA is well suited for biosensing applications because of their specific and robust base-pairing interactions between complementary sequences [58-59].

Exploiting DNA's “code” to bind to a particular molecule inevitably led to the field of aptamers [60-61]. The term aptamer is derived from the Latin 'aptus' meaning "to fit" and is based on the strong binding of nucleobase sequences to specific targets based on structural conformation [62-64]. Aptamers can be both single-stranded RNA or DNA oligonucleotides, typically 15 to 60 base in length that fold into secondary and tertiary structures and bind with high affinity to specific molecular targets. In addition to the genetic information encoded by nucleic acids, aptamers also function as highly specific affinity ligands by molecular interaction based on the three dimensional folding pattern. The three dimensional complex shape of a single stranded oligonucleotide is primarily due to the base composition led intra-molecular hybridization that initiates folding to a particular molecular shape. This molecular shape assists in binding through shape specific recognition to its targets leading to considerable three dimensional structure stability and thus the high degree of affinity.

DNA sensors have been used to detect DNA [65-66], proteins [67-68] and even small molecules/ions [69-70] in the form of optical [65, 71-72], electrochemical [73-75], or mass-sensitive [76-77] for a variety of biological applications. One particular example can be using DNA probes to differentiate healthy, gingivitis, and periodontitis subjects using DNA-DNA hybridization techniques [78]. Most recently, DNA microarray technology has emerged, offering remarkable high-throughput screening properties and reliable biomedical diagnostics applications.
[79-81]. It provides a discovery platform of functional genomics [82-83] and a revolutionizing way of drug design and disease diagnostics [84-85]. DNA sensors are envisioned to be valuable, easy, inexpensive, fast and specific techniques in many applications such as medical diagnostics, genetic screening, drug design, food and agricultural analysis, environmental monitoring and health surveillance. In addition, DNA has been shown to be compatible with other emerging nanomaterials such as carbon nanotubes [38, 86-87], enabling the potential exploitation of the benefits of both materials on simple devices.

DNA can nonspecifically bind to the sidewalls of SWNTs through hydrophobic interactions, π-π bonding [88], and possibly amino-affinity (Fig. 1.3). The aromatic structures in oligomers' bases bind to the aromatic structures on SWNTs via the π-π stacking interactions.

Fig. 1.3. DNA functionalization onto SWNT. a) schematic illustration of DNA double helix structure and its complementary binding properties; and molecular modeling of single-stranded DNA wrapping on SWNT: b) the right-handed helical structure (one of several binding mechanisms including left-handed helices and linearly adsorbed structures) while in all cases, the bases (red)
orient to stack with the surface of the nanotube and extend away from the sugar-phosphate backbone (yellow); c) side-view of the DNA wrapping on SWNT [88].

The single-stranded DNA (ssDNA) functionalized SWNT system offers an intriguing combination of properties of an essential and ubiquitous biomolecule-DNA and one of the most heralded inorganic nanomaterials-SWNT. It has integrated the selective odorant interactions of ssDNA [89] with the sensitivity of SWNTs to the changes in its surface electronic environment when exposed to analytes [90]. Moreover, the response of these devices to a particular chemical of interest can always be enhanced selecting the optimal ssDNA sequences. Functionalization of SWNTs with DNA has demonstrated significant prospects in various fields including the detection of chemical vapors, solubilization in aqueous media, and the nucleic acid sensing [38, 91-92].

DNA decorated SWNTs have illustrated great potential in a variety of fields, ranging from homeland security to disease diagnosis. Aravind et al. have successfully fabricated ssDNA immobilized, Platinum (Pt) nanoparticles decorated MWNT composites to selectively detect dopamine, which is one of the most important neurotransmitters that affect the function of brain [93]. Their hybrid biosensors decorated with sequence AC (bases adenine and cytosine) of ssDNA exhibit linearity of detection up to 0.45 μM and a detection limit about 0.8 μM towards dopamine, while the GT (bases guanine and thymine) sequence of ss-DNA demonstrates linearity of detection to 800 μM and detection limit about 0.45 μM. Moreover, their group’s nafion coated DNA-decorated MWNT biosensor has achieved much better sensing results including good stability, short response time (< 3s) and selective detection of dopamine even with presence of ascorbic acid and uric acid. Apart from the medical field, such novel hybrid nanostructures have found potential applications in the security maintenance of public places by the detection of explosives. Staii et al. have conducted some study on this topic [38]. The odor responses of the ssDNA/SWNT-FET sensors measured were towards DNT and DMMP, which are simulants for explosive vapor and nerve gas, respectively, showing prospects in the security safeguard. Their sensors could detect various odors with very short response time and the recovery time on the scale of seconds. In
addition, the sensor surface can refresh in an ambient environment, enabling the samples to maintain a constant response through at least 50 gas exposure cycles.

In the field of electronic devices, Chen et al. have successfully integrated ssDNA-decorated SWNT-based chemical sensors into CMOS circuitry, which showed great promise towards the development of ultra-small electronic nose [94]. SWNTs were assembled onto the CMOS circuitry by a low AC voltage DEP process, and ssDNA was non-covalently decorated on SWNTs in a humid environment. The decoration of ssDNA on SWNTs was found to increase the resistance of SWNTs by approximately 57.02%. A dramatic enhancement of the sensing response of the gas sensor obtained by decorating ssDNA on SWNT (up to ~300% for methanol vapor and ~250% for isopropanol alcohol vapor) as compared to bare SWNTs has been demonstrated. Liu et al. have developed a single chip nanosensor composed of SWNT integrated on CMOS circuitry with custom designed on-chip amplifiers for chemical agent detection [95]. The SWNTs were integrated on CMOS circuitry using a low temperature and low voltage Dielectrophoretic (DEP) assembly process. Different sequences of DNA were incorporated onto SWNTs and demonstrated improvements of their response to DMMP by 9 times and DNT by 12 times. The responses are reversible and the change in resistance correlated well with the change in concentration of analytes. Not only does this single chip of SWNT sensors provide an attractive platform to realize high sensitivity, portable and compact nanosensing clusters, but it also shows great promise for toxic and explosive gas detection. Thus, it becomes very powerful to apply DNA functionalized SNWTs in chemical and biological sensing.

1.2.2 FUNCTIONALIZATION OF CARBON NANOTUBES WITH POLYMERS

Functionalization of carbon nanotubes with biopolymer chitosan which has excellent film-forming ability, high water permeability, good adhesion, and susceptibility to chemical modifications due to its reactive amino and hydroxyl functional groups, the CNT-chitosan system represents a new biocomposite platform which can be further decorated with enzymes,
nanoparticles, or other biomolecules for various biosensing applications [47-48, 96-97]. In such a system, CNT provide signal transduction while chitosan serves as a biocompatible and chemically modifiable scaffold for immobilization of functional materials such as enzymes, nanoparticles, polymers [98-100].

Reddy’s group has reported a synthesis method of conducting polyaniline-functionalized MWNTs containing noble metal (Au or Ag) nanoparticles composites for applications in nanotechnology, gas sensing and catalysis (Fig. 1.4c) [47]. As illustrated in Fig. 1.4a and b, 10-15 nm diameter of the Au nanoparticles were individually distributed in the functionalized MWNTs composites.
There are so many chemical and electrochemical functionalization methods for CNTs to possess the functional groups that are needed (Fig. 1.5) [101]. It has significantly extended the application spectrum of CNTs into building nanoelectronics and novel biomaterials, biochemical sensing, drug delivery and tissue engineering.

![Possible reactions for the functionalization of the nanotube sidewall](image)

**1.3 SALIVA AND BREATH ANALYSIS**

Saliva has also been long studied to look for possibility in clinical practice. This fluid contains mainly water (99.5%), proteins (0.3%) and inorganic and trace substances (0.2%) [102-108]. The proteins are mostly constituted by glycoproteins, enzymes, immunoglobulins, and many peptides like cystatins, statherin, histatins, proline-rich proteins with antimicrobial activities [103]. The 0.2% inorganic fraction of saliva contains electrolytes of the body fluids such as sodium,
potassium, chloride and bicarbonate, minerals, buffers. The biochemical and physical chemical properties of these salivary components and their interactions can serve to detect systemic disease or evidence of exposure to various harmful substances, as well as provide indications of health and disease status. Moreover, it can also provide information not readily available from serum testing.

Saliva can be accessed through a simple and noninvasive (safe and pain free) collection method, which makes it possible to monitor several biomarkers in infants, children, elderly and non-collaborative subjects, and in circumstances where blood and urine sampling is unavailable. There are good relations between saliva and serum levels of many chemicals which indicate certain organ dysfunctions or diseases (Table 1.2) and this makes the saliva analysis more effective and accurate.

Table 1.2. Saliva as an alternative diagnostic method (compared to serum).

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Indication</th>
<th>No. of specimens</th>
<th>Correlation value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormone</td>
<td>Estradiol (total serum vs. saliva)</td>
<td>Ovarian function</td>
<td>14</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>Progesterone (total serum vs. saliva)</td>
<td>Menstruate function</td>
<td>96</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>Estriol (unconjugated)</td>
<td>Fetoplacental Function</td>
<td>24</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Cortisol (free)</td>
<td>Stress</td>
<td>93</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>17-OH progesterone (total serum vs. saliva)</td>
<td>Menstruate function</td>
<td>13</td>
<td>0.98</td>
</tr>
<tr>
<td>Other metabolism products</td>
<td>Creatinine</td>
<td>Renal function</td>
<td>142</td>
<td>0.967</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>Diabetes</td>
<td>160</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Breath provides insight into the physiological and pathophysiological processes in patients’ bodies, e.g. the sweet smell of acetone accompanies diabetes [116-118]. Breath analysis, as a diagnostic technique, is non-invasive, painless, agreeable to patients, achievable in real time, and can even provide information beyond conventional analysis of blood and urine [119-120]. Many
different analytical techniques were used to analyze exhaled breath, such as gas chromatography and mass spectrometry (GC and MS) [121-122].

The main components in human breath are nitrogen, oxygen, carbon dioxide, water and inert gases. The remaining very small percentage of human breath consists of trace amount of many exogenous molecules and endogenous compounds. Exogenous molecules, particularly those halogenated organic compounds can be used to analyze the environmental and expositional issues while the endogenous compounds can be determined to monitor metabolic or any pathologic processes in the body. The endogenous compounds include inorganic gases such as NO, CO, volatile organic compounds (VOCs) such as ethane, pentane, acetone, isoprene, and other nonvolatile substances such as isoprostanes, peroxynitrite or cytokines. Take lung cancer detection for example, Amann et al. summarized some of the significant VOCs that were used to identify lung cancer (Fig. 1.6) [123]. It is thus possible to diagnose lung cancer through determination of the existence and levels of the breath biomarkers. VOCs can provide great insights into different biochemical processes in either healthy or diseased human bodies.
Both saliva and breath analysis can provide remarkable insights into individual’s health conditions, but we would need the detection of several biomarkers from one analysis method to be reliably determine people’s health status. When we combine two analyzing methods, the complementary nature of these two technologies will reduce the number of biomarkers that are required to detect a disease at an early stage and for health monitoring. Thus, we would be able to clinically monitor a person’s health status, disease progress, and treatment results through the use of an integrated breath and saliva sensor system.

1.4 SCOPE OF THIS THESIS

The motivation of my research work was to develop a noninvasive disease screening, diagnosis and monitoring technology which is crucial to help improve the life quality of many
people. The current clinical practice is either done at clinics which requires sophisticated machines, highly trained professionals, and very expensive, or with portable devices which needs finger pricking and may cause blood borne infections. They both lead to fewer tests and possible late diagnosis, which in turn results in more complications from the disease and significantly increased case management costs, or even deaths. In order to encourage people to check their health status more frequently or monitor their disease progress and treatment results, a noninvasive, cost effective, easy to use, fast in response, and portable sensor technology is highly needed. Knowing there are good correlations between many disease biomarkers or health indicators in saliva and/or breath with blood, we started to develop saliva and breath sensing systems to noninvasively detect biomarkers in saliva and in breath.

I introduced a highly sensitive on-chip glucose sensor built by functionalizing SWNT with gold nanoparticles and glucose enzyme in Chapter 2. Excellent detection results have been obtained. Then we conducted several preclinical trials to detect and monitor the glucose levels in the saliva of healthy subjects. The sensor system was proved to be very clinically accurate. The details of the clinical trials, testing results and insights gained are presented in Chapter 3. Additionally, the dissertation was continued with the investigation of the possibility of detection of other protein biomarkers in saliva through electrochemical method, and some primary results shown in Chapter 4. Besides, DNA-functionalized SWNT sensor array for breath analysis was developed by functionalizing SWNT with several different DNA sequences to detect breath biomarkers through pattern recognition and it was well illustrated in Chapter 5. We also did study on the effect of DNA sequence and length on the sensing performance of this sensor array in Chapter 6, proving the sensing performance is highly affected by DNA sequence and length. One major fundamental obstacle is to find a specific DNA or RNA sequence and length to react and bind with a specific chemical or biomarker. In order to select the best/optimal DNA sequence for certain chemical detection, we used full atomistic molecular dynamics (MD) as a high resolution “virtual microscope”
to characterize DNA/biomarker interactions with high fidelity. In this way, we successfully ranked the strength of the interactions between molecular biomarkers and DNA nucleobases, shown in Chapter 7. As last, I briefly summarize my work and present some future directions for further research.
CHAPTER 2

ON-CHIP ULTRA-SENSITIVE GLUCOSE SENSING USING MULTILAYER FILMS COMPOSED OF SWNT-GOLD NANOPARTICLES-GLUCOSE OXIDASE

It is very important for human health to rapidly and accurately detect glucose levels in biological environments, especially for diabetes mellitus. We proposed a simple, highly sensitive, accurate, convenient, low-cost, and disposable glucose biosensor on a single chip. A working (sensor) electrode, a counter electrode, and a reference electrode are integrated on a single chip through micro-fabrication. The working electrode is functionalized through a layer-by-layer (LBL) assembly of single-walled carbon nanotubes (SWNT) and multilayer films composed of chitosan (CS), gold nanoparticles (GNp), and glucose oxidase (GOx) to obtain high sensitivity and accuracy.

The glucose sensor has following features: 1) direct electron transfer between GOx and the electrode surface; 2) on-a-chip; 3) glucose detection down to 0.1 mg/dL (5.6 µM); 4) good sensing linearity over 0.017-0.81 mM; 5) high sensitivity (61.4 µA/mM-cm²) with a small reactive area (8
mm²); 6) fast response; 7) high reproducibility and repeatability; 8) reliable and accurate saliva glucose detection. Thus, this disposable biosensor will be an alternative for real time tracking of glucose levels from body fluids, e.g. saliva, in a noninvasive, pain-free, accurate, and continuous way. In addition to being used as a disposable glucose biosensor, it also provides a suitable platform for on-chip electrochemical sensing of other chemical agents and biomolecules for disease diagnosis and monitoring.

2.1 INTRODUCTION

According to IUPAC (International Union of Pure and Applied Chemistry) definition, “A biosensor is a self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is in direct spatial contact with a transducer element. A biosensor should be clearly distinguished from a bioanalytical system, which requires additional processing steps, such as reagent addition” [124]. Biosensors can be categorized in terms of the basic principles of signal transduction and biorecognition elements. According to the transducing elements, biosensors can be classified as electrochemical, optical, piezoelectric, and thermal sensors, and the electrochemical ones can be further categorized into potentiometric, amperometric and conductometric sensors. The advantages of biosensors include but not limited to the following perspectives: 1) useful for the detection of nonpolar molecules that do not respond to most other measurement devices; 2) highly specific; 3) rapid and continuous control; 4) fast response; 5) wide sensitive range; 6) high accuracy; and 7) practical. Thus, they have been widely used in areas such as clinic, diagnostic, medical applications, process control, bioreactors, quality control, agriculture and veterinary medicine, bacterial and viral diagnostic, drag production, control of industrial waste water, mining, and military defense industry [125].
Fig. 2.1. Examples of the applications of biosensors [125].

Being able to rapidly and accurately detect glucose levels in biological environments is of crucial importance to human health, especially in the condition of diabetes mellitus [126-127]. In the past several decades, various methods have been developed to measure glucose concentrations, including optical approaches (infrared (IR) spectroscopy, fluorescence spectroscopy, Raman spectroscopy, optical polarization rotation measurement, photo-acoustic probes, and surface plasmon resonance) [128-129], MEMS affinity sensing [130] and electrochemical methods [131-132]. Optical measurement approaches normally require very expensive instruments, significant processing time and highly trained professions; and affinity sensing method can run into nonspecific binding issues. It is the electrochemical glucose sensing that is most studied and demonstrated with high sensitivity, good accuracy, high selectivity, fast response time, low cost and many other outstanding properties [131, 133].

Glucose biosensors have dominated today’s biosensor market. In 2004, approximately 85% of the global market for biosensors was glucose sensors [134]. Glucose sensing dates back to 1841 when glucose levels were measured through urine, but the correlation between urine and plasma glucose was later found inconsistent [135]. Until now, the monitoring of blood glucose levels has been the only recognized and widely used method for diagnosis and management of diabetes. Many kinds of blood glucose meters are available on the market. However, users have to prick their
fingers multiple times a day to use these devices. Repeated painful finger sticks are a major problem for young children and result in negative consequences for disease management. Furthermore, finger pricking can cause transient discomfort, bruise, fainting and blood-borne infection. A noninvasive and simple technique for diagnosis and monitoring of diabetes is thus very desirable. With a direct correlation between blood glucose and salivary glucose, it is possible to simply apply salivary glucose measurements to monitor individual’s health conditions [115, 136]. Hence, monitoring of salivary glucose levels can be an alternative prediagnostic method for diabetics and a health indicator for any individuals.

We have developed a simple, highly sensitive, accurate, convenient, low-cost, and disposable glucose biosensor on a single chip [137]. It can effectively and reliably determine glucose concentrations in saliva. It is fabricated using micro-fabrication and LBL assembly procedures.

2.2 IMMOBILIZATION TECHNIQUES

The effective immobilization of enzymes onto the electrode surface has been one of the main factors that affect the sensing performance of an enzyme biosensor [138]. LBL assembly technique, among many enzyme immobilization methods, is proven to be a simple and effective method to prepare multilayer films containing the enzymes [139]. LBL-assembled multilayer films on the electrodes exhibit good uniformity, stability, reproducibility and remarkable sensitivity [140].

Carbon nanotubes (CNTs) have been widely utilized as components for nanoscale electronic devices and biosensors due to their high electrocatalytic property, their ability to promote electron transfer, and their high thermal capacity. Moreover, the immobilization of CNTs has created a 3-dimentional, porous, conductive catalytic matrix on an electrode surface. The CNTs-modified electrodes are reported to allow direct electron transfer (DET) to glucose oxidase [141]. DET is desirable because it not only provides efficient transduction of enzymatic recognition of an analyte but also eliminates the need for a co-substrate to complete the catalytic cycle.

Chitosan, a linear polysaccharide, is a natural polymer product. It has a repeating hexosamide
residue unit of one amino group and two hydroxyl groups permitting chemical modifications. It is nontoxic, biocompatible and economic. Chitosan is widely applied to immobilize biomolecules, especially in the assembly of enzymes and fabrication of amperometric biosensors, due to its excellent film forming and adhesion abilities, and its easiness for chemical modifications [142-143].

Gold nanoparticles are very attractive in constructing electrochemical biosensors due to their unique physical and chemical properties. In particular, GNp are applied in electrode functionalization to catalyze electrode chemical reactions and conduct DET, and thus to increase the sensitivity of biochemical detection [144]. They are used as one negatively charged nanomaterial for electrostatic adsorption in LBL process. GNp not only increase the surface area to allow more enzyme to be immobilized, but also provide a mild microenvironment and give the biomolecules more freedom in orientation [145].

Tremendous work has been done to develop highly selective and sensitive glucose electrochemical biosensors (Table 2.1). Glucose biosensors functionalized with CNTs or GNp have the ability to linearly detect glucose down to very low levels [146-151]. Chitosan, or polymers like poly(diallyldimethylammonium chloride) (PDDA), poly(ethylenedioxythiophene) (PEDOT), has been commonly used in enzyme immobilization or as a linker molecule in the fabrication of amperometric biosensors [131, 146-147, 149-152]. However, we have achieved much higher sensitivity, good selectivity, good stability and fast response by the integration of nanomaterials-SWNT, GNp, CS, GOx, and an effective film-preparation method-LBL.

Table 2.1. Comparison of the glucose sensing performance of various glucose biosensors

<table>
<thead>
<tr>
<th>Glucose Biosensor</th>
<th>Linear Range /mM</th>
<th>Sensitivity /µA mM⁻¹ cm⁻²</th>
<th>LOD /µM</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCNT/GOx/GAD</td>
<td>6.3-20.09</td>
<td>2.47</td>
<td>-</td>
<td>[132]</td>
</tr>
<tr>
<td>GCE/CNT/Au/PDDA-GOx</td>
<td>0.5-5</td>
<td>3.96</td>
<td>-</td>
<td>[146]</td>
</tr>
<tr>
<td>GNp/MWNTs/GOx</td>
<td>up to 9</td>
<td>7.3</td>
<td>128</td>
<td>[153]</td>
</tr>
<tr>
<td>[GOx/PDDA]₃/[SDS-]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
MWCNT/PDDA$_3$/MPS/Au/Ti/PET & 0.02-2.2 & 5.6 & 10 & [147] \\
GCE/CNT/PTBO-GOx & 1.0-7 & 14.5 & - & [154] \\
ITO/PEDOT/GOx/Nafion & 0.1-1 & 14.06 & 10 & [131] \\
Au/dithiol/au/cystamine/GOx & 0.02-5.7 & 8.8 & 8.2 & [155] \\
ITO/(PEDOT/PSS+PVA)/GOx/PDA & up to 18 & 9.33 & 0.25 & [152] \\
Au/(GOx/GNPs)$_n$ & 0.01-13 & 5.72 & - & [148] \\
Au/MPS/TH/(SCGNPs/TH)$_n$ & up to 3 & 3.8 & 35 & [156] \\
GCE/CS/MWNTs-Fc/GOx & 0.012-3.8 & 25 & 3 & [149] \\
Pt/PAA/GNps/GOD/CS/GNPs/GOx & 0.5-16 & - & 7 & [150] \\
GCE/CS-PB@MWNTs/HPtCo/GOD/Nafion & 0.003-3.6 & 21 & 0.85 & [151] \\
Pt/PAA/SWNT/(CS/GNp/GOx)$_3$ & 0.017-0.81 & 61.43 & 5.6 & our work \\

Additionally, most of the other research work focused only on the functionalization of sensor electrode regardless of the electrode size or configuration which highly affects the simplicity, convenience, and commercialization possibility of the biosensors. Conventional electrochemical tests are conducted in standard electrochemical cells consisting of a working electrode (e.g. a modified glassy carbon electrode (GCE)), a counter electrode (e.g. Platinum (Pt) wire), and a reference electrode (e.g. Ag/AgCl). It is restricted to a standard lab test setup including a beaker containing 10-20 mL sample solution with three electrodes immersed in it and a stirring system as optional. Therefore, it is impractical to monitor glucose levels of individuals through their body fluids, like saliva, using this test setting.

We have developed an on-chip electrochemical sensing device with at least one working electrode, a counter electrode and a reference electrode. It is manufactured through several microfabrication procedures. The metal for all electrodes is Pt, which is widely applied for glucose sensing [143]. Pt provides significant advantages, such as much better conductivity, signal stability, and analytical response, over the other electrode materials, like Au, Ti, Ag [157-158]. Jin et al. has also proved that Pt’s deposition onto the gold electrode can increase the electrocatalytic properties
of the electrodes for glucose oxidation [159]. Pt can also be used for reference electrode [160]. Thus, it is highly feasible to integrate three Pt electrodes onto one single chip to realize on-chip electrochemical sensing. Further, this single chip can not only be used for glucose detection, but also provides an innovative platform for on-chip electrochemical sensing of other chemicals and biomolecules.

### 2.3 EXPERIMENTAL PROCEDURES

Fabrication of the disposable glucose biosensor is described in this section. It includes microfabrication of the sensor chip and LBL assembly for electrode modification.

The on-chip electrochemical sensing device contains at least one working electrode, a counter electrode and a reference electrode (one possible electrode configuration - Fig. 2.2a). The small rectangle marks out the reactive area on the working electrode while the larger one indicates where sample drops on. One such device – S2D2 is of size 20×10mm² with the reactive area 32mm². It can be manufactured through microfabrication (Fig. 2.2b). Starting from a pre-cleaned silicon wafer (~500 µm thick), the surface was oxidized in wet atmosphere (Bruce Furnace 7355B) at 1100 °C for 40 minutes to form a 0.5µm thick SiO₂ layer as the insulator layer. Then, photolithography is conducted to create the pattern of desired microelectrodes using MICROPOSIT S1813 photoresist. 200 nm thick Pt (Platinum) with 20 nm thick Cr (Chromium) adhesive layer is deposited on the surface through E-beam evaporation. After lifting off the extra Pt by acetone, isopropyl alcohol (IPA) and DI water and dicing the wafer into small chips (20×10mm²), the electrode system was present on each chip.
Fig. 2.2. a) Schematic diagram of the electrochemical system and the sensing area on a chip; b) micro-fabrication procedures of the microelectrodes on a silicon wafer.

The glucose biosensor is fabricated through a LBL assembly of SWNT and multilayer films composed of CS-GNp-GOx (Fig. 2.3) [161]. The CS-GNp-GOx unit can be repeated several times to form a multilayered coating. The number of layers can be adjusted to achieve the best sensing performance.
2.4 RESULTS AND DISCUSSION

2.4.1. SENSOR CHARACTERIZATIONS

Poly (allylamine) (PAA) was adsorbed onto the Pt electrode surface non-covalently with its amino radicals (highly positively charged) [162]. SWNT with carboxyl groups at both ends connected PAA and chitosan through covalent bonds. SWNT, represented by the short white lines
(about several hundred-nm long), was successfully assembled on the electrode surface (Fig. 2.4a). Despite some white dots, which were believed to be aggregated SWNTs or small containments, the overall coating uniformity was very good. GNp was assembled on CS through electrostatic interaction [163] and was uniformly distributed with minor amounts of aggregation (diameter of GNp is 20nm) (Fig. 2.4b). SWNT could be clearly observed underneath the GNp layer, and the dark flocculent areas were potential chitosan molecules connecting SWNT with GNp. Finally GOx was adsorbed onto the surface of GNp through the –NH₂ group; while the negatively-charged characteristics allowed GOx to assimilate the polycationic CS by electrostatic adsorption. With one layer of GOx assembled on the electrode surface there were some scattered molecular clusters probably because of insufficient GNp sites for GOx molecules to bind with or space repulsive force between GOx molecules (Fig. 2.4c). After assembling two more layers of GOx, the accumulated layers have successfully covered the entire reactive area and the pattern was well guided by the SWNT underneath (Fig. 2.4d).
Our glucose sensor detects glucose levels by keeping track of the electrons passed through GOx to the electrode surface. The charge transfer complex formed by the functional layers of SWNT-CS-GNp between glucose oxidase and the electrode surface has permitted direct electron transfer between the active center of glucose oxidase and the electrode. With more effective electrical communication between enzyme and the electrode surface, it has remarkably improved the sensor selectivity, sensitivity and stability.

Fig. 2.4. Cross-section of the functional layers on the sensor electrode and SEM images of a) one layer of SWNT; b) one layer of SWNT/GNp; c) PAA/SWNT/CS/GNp/GOx film; d) PAA/SWNT/(CS/GNp/GOx), film on the sensor electrode surface.
2.4.2. GLUCOSE SENSING

Cyclic voltammetry (CV) measurements were conducted to detect glucose of different concentrations in PBS. Each 200 µL sample was dropped onto the sensing area (Fig. 2.5a). Current response increased with the elevation of glucose concentration, and tended to reach a saturation value at high glucose concentration where all active sites of GOx were taken up. It agreed with the characteristics of Michaelis-Menten kinetics. The reason our applied potential was different from other research groups is due to the difference in the sensor functionalization method and the reference electrode material (ours is Pt while others is Ag/AgCl or SCE). A linear sensing ability at least over the range of 1-200ppm (0.017-1.11 mM) with a sensitivity - 26.6 µA/mM-cm² and correlation coefficient of 0.995 was revealed (insert of Fig. 2.5b).

![Graph](image)

**Fig. 2.5.** Glucose sensing using the on-chip electrochemical sensing system S2D2 by, a) cyclic voltammetry of Pt electrode modified with SWNT/(CS/GNp/GOx)₃ layers obtained in 0-400ppm glucose solutions with the voltage between WE and RE ranges from -0.4 - 0.4V at a scan rate of 50 mV/s; b) steady-state calibration curve of the SWNT/(CS/GNp/GOx)₃ functionalized Pt electrode at applied potential 0.2V with linear detection range shown in the inset. Error bars = ± standard deviation and n = 3.

We compared the amperometric test results at 20s point and at a time window of 18-21s (Fig. 2.6a and b). Four individual sensors were used to measure each glucose concentration. Compared
to data at single time point, the integration one demonstrated better repeatability with excellent linearity over 5-200 ppm. Thus, we continued using current integration at applied potential-0.2V as our data analysis method.

![Fig. 2.6. Amperometric measurements of different-concentration glucose solutions at applied potential 0.2V with a) output current at 20s; b) integrated current in 18-21s after data processing. Error bars = ± standard deviation and n = 4.](image)

Furthermore, we microfabricated another two types of sensing chips with 1) the same configuration but with ¼ size of the original one (system 0.25); 2) a round configuration (system r) (Configuration in Table 2.2) and decorated each with GOx using the same fabrication procedures. The aim was to prove the reproducibility of our fabrication procedures, and more importantly, to accomplish higher sensitivity with much smaller size and less sample volume. The required sample volume has been reduced greatly from 200 µL to 40 µL for system 0.25 devices. The round shape device with a 12 mm² reactive area was chosen because one drop of glucose sample is sufficient to cover the whole electrochemical sensing area which makes the test preparation more convenient. The glucose sensing performance of these different sensor systems was summarized in Table 2.2. Reducing the sensor size can greatly increase the sensitivity due to the increased electron transfer efficiency. System r provided the highest sensitivity for glucose detection. The reason is very likely to be the much higher electron transfer efficiency achieved by the much shorter distances between working and reference electrodes and between working and counter electrodes, and the more
uniform electron distribution as well.

Table 2.2. Sensing performance of different sensor configurations.

<table>
<thead>
<tr>
<th>Configuration</th>
<th>Size (mm²)</th>
<th>Reactive area (mm²)</th>
<th>Sample size (µL)</th>
<th>Detection limit (µM)</th>
<th>Linear detection range (mM)</th>
<th>Sensitivity (µA/mM-cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>system S2D2</td>
<td>200</td>
<td>32</td>
<td>200</td>
<td>11.1</td>
<td>0.017-1.11</td>
<td>26.6</td>
</tr>
<tr>
<td>system 0.25</td>
<td>50</td>
<td>8</td>
<td>40</td>
<td>5.6</td>
<td>0.017-0.81</td>
<td>61.43</td>
</tr>
<tr>
<td>system r</td>
<td>200</td>
<td>12</td>
<td>100</td>
<td>16.7</td>
<td>0.027-0.56</td>
<td>69.9</td>
</tr>
</tbody>
</table>

Thus, we have verified the high reproducibility and reliability of our glucose sensor fabrication procedures and demonstrated the electrochemical sensing system’s ultra-high sensitivity, fast response, and great repeatability. The sensor can also be miniaturized and optimized in size or configuration for other desired applications.

2.5. CONCLUSION

In summary, we have developed a simple and economic on-chip electrochemical sensing system containing at least one working electrode, a counter electrode and a reference electrode. It is now used as a disposable nano-biosensor for glucose detection with the sensor electrode functionalize with SWNT and multilayered film composed of CS-GNp-GOx. It can detect glucose down to 0.1 mg/dL with good sensing linearity over range of 0.017-0.81 mM and has an ultra-high sensitivity of 61.425 µA/mM-cm² with a small surface area 10×5 mm². It can also be further miniaturized and maintain high reproducibility and repeatability. It is an alternative for real time tracking of glucose levels from body fluids, e.g. saliva, in a noninvasive, pain-free, accurate and continuous way. Furthermore, this on-chip electrochemical system is a platform suitable for on-chip electrochemical sensing of other chemical agents and biomolecules.
CHAPTER 3

NONINVASIVE GLUCOSE MONITORING USING SALIVA NANO-BIOSENSOR

Millions of people worldwide live with diabetes and several millions die from it each year. A noninvasive, painless means of glucose testing would highly improve compliance and glucose control while reducing complications and overall disease management costs. To provide such an accurate, low cost, and continuous glucose monitoring method, we have developed a unique, disposable saliva nano-biosensor. More than eight clinical trials on real-time noninvasive salivary glucose monitoring were carried out on two healthy individuals (a 2-3 hour-period for each trial, including both regular food and standard glucose beverage intake with more than 35 saliva samples obtained). Excellent clinical accuracy was revealed as compared to the UV spectrophotometer. By measuring subjects’ salivary glucose and blood glucose in parallel, we found the two generated profiles share the same fluctuation trend but the correlation between them is individual dependent. There is a time lag between the peak glucose values from blood and from saliva. However, the correlation between the two glucose values at fasting is constant for each person enabling
noninvasive diagnosis of diabetes through saliva instead of blood. Furthermore, a good correlation of glucose levels in saliva and in blood before and two hours after glucose intake was observed. Intervals before and two hours after meals are usually prescribed by doctors for diabetic patients. Thus, this disposable biosensor will be an alternative for real-time salivary glucose tracking at any time.

3.1 INTRODUCTION

The International Diabetes Federation estimates 382 million people worldwide had diabetes in 2013, and the number is forecasted to reach 592 million by 2035 (a 55% increase) [164]. There were 5.1 million diabetes-related deaths globally in 2013, equaling to one death every six seconds, an 11% increase over 2011 [165]. Early diagnosis, on-time treatment and continuous management are vital to patients’ life quality and to avoid complications such as circulatory problems, kidney failure, heart disease, stroke, and blindness [166-167]. Current practices for diabetes management rely on monitoring blood glucose levels. Blood glucose measurements are required to determine insulin dosage and to detect abnormal glucose levels indicating illnesses, dietary changes, or adverse medication responses. These intrusive tests are generally disliked because of the pain and inconvenience caused by finger pricking, resulting in fewer tests and inadequate blood glucose control. Poor blood glucose control results in more complications and even higher management costs. Particularly, repeated painful finger sticks are a major problem for young children and result in similar negative consequences for disease management.

Glucose sensing started in 1841 when it was performed in urine, but unfortunately the correlation between urine and plasma glucose was inconsistent [135]. Monitoring of blood glucose levels is currently the only recognized and widely used method for the diagnosis and control of diabetes. There are many different types of blood glucose meters on the market; however, they all require users to prick their fingers multiple times a day to obtain blood samples. Some minimally invasive or noninvasive techniques for blood glucose monitoring were studied, including infrared
(IR) spectroscopy, fluorescence spectroscopy, Raman spectroscopy, and surface plasmon resonance. However, the results still have to be correlated with direct blood glucose measurements, and the sensitivity and reliability are limited by spectral signal-to-noise level and skin thickness. For example, in 2002, Cygnus Inc. introduced a wearable GlucWatch device measuring the glucose electroosmotically extracted across skin [168]. Nevertheless, the difficulty of use due to the sweat collection process and the low level of accuracy resulted in its removal from the market. Another product, the OrSense NBM device provided by OrSense Ltd [169], which detects blood glucose concentration via an optical method called "occlusion spectroscopy" [170], has not achieved any significant success. Although optical technologies for glucose determination are available, most of them are for laboratory use due to the size, cost, and complexity of operation. Thus, a noninvasive, convenient, accurate, easy-to-use, portable, and low-cost diagnostic tool for diabetes is highly demanded.

As summarized by Lei et al. [171], there are three necessary prerequisites for most clinical applications: i) a simple and inexpensive method for collecting biological samples with minimal discomfort, ii) specific biomarkers associated with health or disease, and iii) an accurate, portable and easy-to-use technology for disease diagnosis and health screening. Saliva, commonly considered as the ‘mirror of the body’, is very attractive as a bio-medium for clinical diagnostics. Its unique properties, such as noninvasive accessibility and the presence of plentiful disease biomarkers, make it particularly attractive for disease diagnosis and monitoring [172-173]. Saliva can be easily collected by individuals with modest instruction and it dramatically reduces the discomfort of the tests. Changes in saliva are believed to indicate the wellness of an individual. There are a large number of diagnostic analytes present in saliva, including glucose [174-175], steroid hormones [176], and the HIV antibody [177]. Saliva was first demonstrated to have diagnostic power comparable to that of blood in differentiating smokers from non-smokers through thiocyanate ions levels [178]. Results from blood, saliva, and urine as biomedia were compared and saliva was recognized as the most sensitive one. Saliva is also revealed to be more accurate
than blood in detecting oral cancer [179-180]. Furthermore, the concentration of some other disease biomarkers in saliva was found to exceed that in blood, illustrating a further advantage of using saliva for clinical diagnostics [181-182].

Regarding to the technologies for determining salivary glucose levels, optical measuring systems such as Liquid Chromatography-Mass Spectrometry (LC-MS) and UV-VIS Spectrophotometry were reported [136, 183]. However, the measurements can only be done in a laboratory as they require significant processing time, expensive reagents, sophisticated instrument, and highly trained professionals. Consequently, these methods cannot be used for individual glucose monitoring at home or in daily activities. Until now, there isn’t a suitable product for home care measurement of glucose using saliva. Technologies, including microchips and microfluidic devices, show great potential in developing a robust, cost-effective, accurate, portable, and easy-to-use diagnostic tool for saliva analysis [173, 184]. Miniaturized saliva-based diagnostic technologies will enable the use of trace amount of biofluids to provide quick and reliable results for clinical decision-making and treatment outcomes-predicting.

A positive correlation between blood glucose and salivary glucose is revealed by many studies [115, 136, 185-187]. Other than salivary glucose, no other parameters in saliva were found to be markedly affected in diabetes mellitus [175]. Therefore, salivary glucose can be utilized as an alternative diagnostic method for diabetes and as a general screen for prediabetes and undiagnosed diabetes.

Here we proposed an on-chip disposable nano-biosensor providing a painless test methodology with sufficient sensitivity. It is disposable and thus eliminates extensive cleaning or electrode pretreatment between measurements. The working electrode is functionalized with single-walled carbon nanotubes (SWNT) and multilayers of chitosan (CS), Gold nanoparticles (GNp) and glucose oxidase (GOx), using a layer-by-layer (LBL) assembly technique [188]. The biosensor can detect glucose down to 0.1 mg/dL and provide noninvasive, reliable (high resolution), highly reproducible, convenient, fast, and continuous salivary glucose monitoring for personal and
3.2 PRECLINICAL TRIAL PROCEDURES

3.2.1. DEVICE FABRICATION

The device manufacturing procedures were the same as in Chapter 2.

3.2.2. SALIVA SAMPLING PROCEDURES FOR TEST SUBJECTS

Two healthy volunteers of age-group 20-30 years were enrolled in this study, following the Northeastern University’s Institutional Review Board (IRB), wherein the individuals signed consent form and received a $12.00 gift card at the completion of each session with their identities unrevealed.

The following protocol was introduced to all subjects and executed in all preclinical tests:

1. Wait for two minutes after rinsing mouth with water;
2. Minimize swallowing and hold saliva in mouth (typically <1 min);
3. Place dental sterilized cotton sponge in mouth and chew until it is soaked with saliva (typically <1 min);
4. Deposit sponge into syringe directly from the mouth without touching it to avoid contamination;
5. Insert plunger into syringe;
6. Squeeze saliva through preinstalled membrane (PVDF membrane) in the bottom of syringe into sterilized tubes gently (approx. 100 µL, per sensor per measurement needed);
7. Preserve sample tubes in 4 °C chill box while producing samples;
8. Take pipette to drop saliva (100 µL) onto glucose sensor;
9. Perform amperometric measurement (30 sec) and display;
10. Dispose sensors after washing out the residue salivary specimen on it.
3.3 RESULTS AND DISCUSSION

3.3.1. SENSOR CHARACTERIZATION

The uniformity of assembled SWNT, GNp, and GOx layers was inspected by SEM (Fig. 3.1). SWNT and GNp were well distributed (Figs. 3.1a and b). Three layers of GOx matrix covered the whole reactive surface on the working electrode (Fig. 3.1d) while one layer could only cover partially the reactive surface (Fig. 3.1c). SWNT, represented by the short white line (about several hundred-nm long), were successfully assembled onto the functional area (Fig. 3.1a). Besides some white dots, which were believed to be aggregated SWNTs or small contaminants, the overall coating uniformity was very good. GNp can be assembled on chitosan through electrostatic interactions [163]. The SEM image (Fig. 3.1b) also proved that GNp were uniformly distributed with a minor amount of aggregation. SWNT could be clearly observed underneath the GNp layer. The dark flocculent areas presented potential chitosan molecules connecting SWNT with GNp. Finally, GOx was adsorbed onto GNp through -NH₂ groups. Fig. 3.1c shows one layer of GOx assembled on the sensor electrode. The scattered molecular clusters were very likely due to the insufficient GNp sites or the space repulsive force between GOx molecules. After assembling two more GOx matrix, the accumulated glucose oxidase successfully covered the whole reactive surface and its pattern was guided by the underlying SWNT (Fig. 3.1d).
Fig. 3.1. SEM images of functional layers of the glucose sensor: a) one layer of SWNT; b) one layer of SWNT/GNp; c) PAA/SWNT/CS/GNp/GOx film on the sensor electrode surface; d) PAA/SWNT/(CS/GNp/GOx)3 films on the sensor electrode surface.

We compared the detection results of glucose in buffer solution using sensors with one layer of GOx and three layers of GOx coating (Fig. 3.2 e and f) and it confirmed sensor functionalized with PAA/SWNT/(CS/GNp/GOx)3 film had much better sensitivity and repeatability. Furthermore, multi-layered GOx coating enhanced the linearity of the sensor response.
Fig. 3.2. Amperometric measurements of glucose buffer solutions at applied potential 0.2 V using sensors functionalized with e) PAA/SWNT/CS/GNP/GOx film; and f) PAA/SWNT/(CS/GNP/GOx)3 film. Error bars = ± standard deviation and n = 3.

Our on-chip glucose sensor determines salivary glucose levels by keeping track of the electrons passed through the glucose oxidase enzyme coated on the working electrode. The charge transfer complex formed by the functional layers of SWNT-CS-GNP between glucose oxidase and the electrode surface has permitted direct electron transfer between the active center of glucose oxidase and the electrode allowing direct oxidation of the enzyme. It eliminates the inherent limitations of redox mediators in bio-electrocatalytic applications [189]. The mediator-free glucose sensing mechanism provides effective electrical communication between enzyme molecules and the electrode surface.

As demonstrated in Chapter 2, the sensor provides a reliable linear detection range over 0.1-20 mg/dL (0.017-1.11 mM) with sensitivity = 26.6 µA/mM-cm² and correlation coefficient of 0.995 [190]. We also selected the low applied potential- 0.2V, which can significantly reduce the possible interference from other electroactive species whose oxidization potentials are close to glucose [191-193].

3.3.2. SALIVARY GLUCOSE MONITORING
Saliva sampling procedure listed in the Experimental section was developed based on our research objectives and referred from some literature on saliva analysis [186, 194-195]. Saliva samples we collected from each participant were all unstimulated which revealed the participants’ real metabolic conditions. It would increase the test accuracy.

Experimental saliva collection and sampling devices were shown in Fig. 3.3. A dental cotton roll was used to soak enough saliva, then a PVDF (polyvinylidene fluoride) membrane was applied to filter out big molecules, and finally the iron wire gauze was applied to fix and stabilize the membrane at the bottom of the syringe. Filtered saliva samples were collected in self-standing screw cap tubes. The samples were then split to two parts. One part was used to immediately determine its glucose content by using our glucose sensors. The other part was boiled, centrifuged and measured by UV Spectrophotometer to assess the accuracy of our sensors.

The particular PVDF membrane we selected has a protein binding capacity over 200 µg/cm² and a pore size of 0.2 µm. It has maximum immobilization of proteins during sample transfers and minimized sample loss. The small pore size also eliminates ‘blow-through’ and increases protein binding over a wide range of molecular weights. Thus, it can filter out most of the proteins in saliva rendering the glucose determination more accurate. Another perspective for selecting the membrane is to decrease the viscosity of saliva samples. Viscosity of human saliva varies between
individuals and is easily affected by the biological environments [196-197]. We kept track of the viscosity of raw saliva and the membrane filtered saliva of one healthy subject with one Trutol® 75 Glucose Tolerance Beverage intake (Fig. 3.4). The subject was asked not to eat or drink anything from 10 pm the night before the test. Approximately 1 mL of each saliva samples was collected before (-30 min) and 60, 120, and 180 min after the glucose beverage intake and measured three times. As demonstrated in the viscosity monitoring results, we managed to successfully reduce the viscosity of saliva via this membrane. The filtered saliva samples had a viscosity of 1.07-1.13 mPa-s which were believed not to affect the glucose detection.

![Fig. 3.4. Viscosity changes with 75g glucose tolerance beverage intake of raw saliva (black); PVDF filtered saliva (blue).](image)

We did sensor calibration with the UV spectrophotometer of 75 fasting saliva samples from two healthy individuals on different days. The subject was asked not to eat or drink anything from 10 pm the night before the test. The fasting saliva samples were collected using the device (Fig. 3.3) and separated to two sections: one part was immediately measured using our sensors and the other part was processed for UV spectrophotometer measurement. The procedures were: the saliva samples were boiled at 100 °C for 30-40 minutes then cooled down to room temperature. After
centrifuging at 12,000 g for five minutes to remove any particulate materials, each supernatant was transferred to new micro-centrifuging tubes and reacted with the glucose assay kit (purchased from BioVision Company). The glucose content of each saliva sample was determined by the absorbance at 570 nm using UV-mini 1240 Spectrophotometer. A clear linear correlation between them was obtained (Fig. 3.5). Therefore, our sensor can be employed to determine the glucose levels in saliva based on the linear correlation.

![Sensor calibration with UV spectrophotometer.](image)

Fig. 3.5. Sensor calibration with UV spectrophotometer.

Based on this, we compared subject A’s blood glucose (BG) measured by blood glucose meter and salivary glucose (SG) measured by our sensors at fasting on different days (Fig. 3.6). There is clearly a constant correlation between BG and SG at fasting state on different days. It permits saliva analysis to be an alternative noninvasive diagnostic method for diabetes and our sensor as a general screen tool for prediabetes and undiagnosed diabetes.
Furthermore, we measured the glucose levels in both fasting and after-meal saliva samples using our sensors and the UV spectrophotometer and did error grid analysis to quantify the clinical accuracy of salivary glucose estimates generated by sensors as compared to UV spectrophotometry method (Fig. 3.7). The subjects were asked to fast overnight and to have a regular meal after taking fasting saliva and blood samples. Several more sets of saliva and blood samples from them were measured after meal. There were 68 saliva samples obtained from two healthy young adults, and the glucose concentrations were between 0.6 to 1.2 mg/dL, in agreement with results from many other clinical studies [185, 187, 198]. Region A are those values within 20% of the reference method, while Region B to E contain points that are outside of 20% would not/ would lead to inappropriate treatments in a severity-level ascending order. With all data falling in Region A, it well demonstrates the salivary glucose values measured by our sensors are within 20% of the reference UV spectrophotometry method, thus, it has a very high clinical accuracy.
Fig. 3.7. Clinical accuracy evaluation of salivary glucose estimated by sensors compared to values obtained from UV spectrophotometer with the zoom-in image as an inset.

Here are some other findings in our glucose monitoring clinical trials for healthy young adults where saliva and blood glucose were measured simultaneously before and after glucose-content food/beverage intake and each sample was measured at least three times:

1. SG value reaches its peak value approximately 15 min-40 min after food intake while the BG increases to its highest value at around 30 min-60 min after. The time difference in reaching peak values is regarded as a normal physical mechanism [136, 185, 198]. After peak, both SG and BG begin to decrease until both drop to normal ranges within 3 hours.

2. Intense physical exercise after food intake can cause both SG and BG concentrations to be very low (e.g. 70 mg/dL) even after 3 hours but still in healthy range.

3. The physiological responses after food intake vary from individuals, and their normal SG and BG ranges can also be different.

4. Different individuals have different carbohydrate metabolism after food intake, and the fluctuations in SG and BG levels also vary for one subject on different days. However, the rise and fall of glucose levels in saliva and blood were observed and the fluctuation in SG
measured by our sensors was in compliance with that in BG. Moreover, the little variance between the SG measured by our sensors and by the UV Spectrophotometer also proved the great accuracy and reliability of our sensing system.

5. Changes of glucose levels both in blood and in saliva are different whether the subjects take regular meals or 75g glucose tolerance beverage. Regular food contains not only sugar (which can be directly digested to glucose) but also protein and fat, and some of the protein or fat will not be turned into glucose unless necessary. Glucose beverage, on the other hand, is just 75g glucose dissolved in water which can be directly transited into blood and into saliva. The difference in body metabolism upon the intake of different types of carbohydrates was reported [136, 174, 185, 198-200]. The mechanism of human carbohydrate metabolism is beyond our knowledge but we will carry out more research to understand these dynamic shifts in both saliva and blood glucose, and develop a better way to interpret salivary glucose levels in real time.

6. A high correlation of glucose levels in saliva as measured by our sensors and blood glucose levels as measured by the standard finger stick technique in healthy volunteers before and two hours after an intake of a 75g glucose containing beverage was observed. Two-hour is a common interval prescribed by doctors for diabetic patients to check blood glucose levels before and after meals. Thus, the high feasibility to use saliva analysis as an alternative, noninvasive, and convenient diagnosis method for diabetes and for glucose monitoring in diabetes patients is well established. Our sensor will allow a convenient and painless determination of equivalent blood glucose levels through salivary glucose monitoring.

3.4. CONCLUSION

We have developed an innovative, simple, and low cost on-chip electrochemical glucose sensing system. It can provide noninvasive, reliable, convenient, fast, and continuous salivary glucose monitoring for personal and point-of-care use. It also demonstrates excellent clinical
accuracy as compared to salivary glucose obtained by UV spectrophotometer. The constant correlation between BG and SG for each person enables noninvasive diagnosis of diabetes through saliva. Although the correlation between BG and SG profiles is highly individual dependent, there is a good correlation between glucose levels in saliva and in blood before and two hours after glucose intake. Thus, this disposable glucose sensor is very powerful for real-time convenient tracking of glucose levels in saliva at any time, and can further be used for both diagnosis of diabetes and glucose monitoring.

The sensor can also be miniaturized and optimized in shape or re-configured for other applications. The sensitivity can be optimized by adjusting the coating procedure for the working electrode and by precise automatic production. The sensor can serve as a stand-alone device, or be incorporated into another device. Furthermore, it provides a good platform for on-chip electrochemical sensing of various other chemicals and biomolecules.
CHAPTER 4

SALIVARY PROTEIN BIOMARKER DETECTION USING GOLD NANOPARTICLES

Saliva has become more and more important as a resource for evaluating physiological and pathological conditions in humans and as a method for the diagnosis of oral and systemic diseases. There are many advantages using saliva, such as the simple and noninvasive collection procedures, easiness, and safety. Human saliva proteome (HSP) can provide great insights into people’s health conditions or disease progresses. Here we presented results of primary testing of fibrinogen indicating diabetes and cardiovascular diseases using our electrochemical sensors functionalized with gold nanoparticles. Approximate linear detection between 0.005-500 mg/dL was obtained in fibrinogen Tris-HCl buffer solution. We proposed to apply anti-fibrinogen antibodies in building the sensors to increase the sensitivity especially for the detection of ultra-low levels of fibrinogen.

4.1 INTRODUCTION
Saliva is a very attractive body fluid for clinical diagnosis and prognosis of human diseases and the noninvasive collection procedures for saliva dramatically reduce anxiety and discomfort (Fig. 4.1). Compared with blood, saliva contains many locally secreted proteins distinct from serum, thus these proteins can serve as better indicators of oral cavity diseases, such as periodontitis [201-203], oral squamous cell carcinoma (OSCC) [204-210] and Sjogren’s syndrome (SS) [211-212]. Besides oral diseases, saliva is also broadly applied in the diagnosis of systemic diseases that affect the function of the salivary glands and the composition of the saliva, such as alcoholic cirrhosis, cystic fibrosis, sarcoidosis, diabetes mellitus and disease of the adrenal cortex. With advances in microbiology, immunology and biochemistry, salivary testing in clinical and research settings is rapidly proving to be a practical and reliable means of recognizing oral signs of systemic illness and exposure to risk factors, and receives growing acceptability.

![Fig. 4.1. Biomarkers present in saliva [213].](image)

For example, salivary markers are explored to noninvasively detect breast cancer in conjunction with mammography. Protein c-erbB-2 is a prognostic breast cancer marker assayed in tissue biopsies of women diagnosed with malignant tumors. The soluble fragments of the c-erbB-
2 oncogene and the cancer antigen 15-3 were reported to be significantly higher in the saliva and serum of women who had cancer than in the saliva and serum of healthy controls and patients with benign tumors [214]. Pilot studies have revealed that the saliva testing for this c-erbB-2 is very sensitive and reliable, thus, it provides high potential to be used in early detection of and follow-up screening for breast cancer [215].

Salivary biomarkers are being explored as a noninvasive and easy means of monitoring general health and in the early diagnosis of disease. For example, the onset and severity of infectious diseases can be determined by monitoring the presence of antibodies to the microorganisms found in saliva and the oral cavity.

Fibrinogen is a soluble plasma glycoprotein, synthesized by the liver, which is converted by thrombin into fibrin during blood coagulation. It may be elevated in any form of inflammation, it is especially apparent in human gingival tissue during the initial phase of periodontal disease. Epidemiologic studies have implicated fibrinogen in the occurrence of cardiovascular disease [216-218]. The study at Framingham revealed that for both women and men, the risk of cardiovascular disease was correlated positively to antecedent fibrinogen values higher than the 126 to 696 mg/dL range [218]. Thus, the elevated fibrinogen level is of great value for prognosis of cardiovascular disease. Moreover, the level of fibrinogen in plasma of diabetic patients was reported much higher than that of non-diabetic people (304 ± 31 vs. 210 ± 12 mg/dL) [219-222].

Deng et al. has reported the binding kinetics of human fibrinogen to negatively charged poly(acrylic acid)-coated gold nanoparticles ranging in size from 7 to 22 nm [223]. Larger nanoparticles were found to be able to accommodate more fibrinogen molecules due to the larger surface area (Fig. 4.2). Using this fibrinogen-gold nanoparticle binding mechanism [223-225], we built our electrochemical sensors with gold nanoparticles, and did fibrinogen testing at a range of 5×10^{-11} to 500 mg/dL in Tris-HCl buffer solution.
4.2 EXPERIMENTAL PROCEDURES

Using system r (Table 2.2) as sensor substrate and assembling a matrix of PAA, SWNT and GNP through a LBL assembly process (Fig. 2.3) to functionalize the sensor with gold nanoparticles. SWNT was applied to increase the electron transfer efficiency and thus shorten the response time and enhance the sensitivity. The diameter of gold nanoparticles is 20 nm, which lies in the range of large surface areas possessing excellent binding properties with fibrinogen. Fibrinogen was disbursed in pH 7.4 Tris-HCl buffer solution to make final concentrations at $5\times10^{-11}$, $5\times10^{-10}$, $5\times10^{-9}$, $5\times10^{-7}$, $5\times10^{-5}$, 0.005, 0.01, 0.1, 0.5, 500 mg/dL. The reason to push the detection at two ends (high and low) is to make sure we can effectively detect extreme levels of fibrinogen which can be vital for patients. The tests were conducted at applied potential 0.2V using the amperometric measurements.
4.3 RESULTS AND DISCUSSION

An approximate linear detection was obtained between concentrations of 0.005 mg/dL and 500 mg/dL (Fig. 4.3). The symbol of the output current is minus, meaning the current was negative, is an indication of the direction of the current. The absolute value of the current reveals the reaction strength which in turn tells about the concentration of fibrinogen. The higher concentration, the more binding between fibrinogen and gold nanoparticles, and it increases the resistance, resulting the decrease of the absolute value of the output current.

Fig. 4.3. Amperometric measurements of fibrinogen solutions at concentrations of 0.005, 0.01, 0.1, 0.5, 500 mg/dL at applied potential 0.2V. Error bars = ± standard deviation and n = 2.

Aiming to push the detection limit at the very low level of fibrinogen, we applied the same amperometric measurements using another batch of SWNT-GNP fictionalized electrochemical sensors to detect fibrinogen from $5 \times 10^{-11}$ to 0.005 mg/dL (Fig. 4.4). Different concentrations at this range could not be distinguished from each other. Therefore this GNP-fibrinogen binding reaction for the detection of fibrinogen is not sensitive enough to serve as a diagnostic method for clinical applications. The proposed solution is to decorate GNP with anti-fibrinogen antibodies on the sensors. Since the binding between antigen and antibody is much more robust and specific, it can highly improve the sensitivity of fibrinogen.
4.4 CONCLUSION

In this Chapter, the detection of protein biomarker-fibrinogen through electrochemical measurements was introduced. Fibrinogen can potentially indicate diabetes and cardiovascular diseases, so it is of high value to be able to reliably detect the levels of it for the applications of health monitoring. Here we presented the primary testing results of fibrinogen using our electrochemical sensors functionalized with single-walled carbon nanotubes and gold nanoparticles. Approximate linear detection between 0.005-500 mg/dL was obtained in fibrinogen Tris-HCl buffer solution. However, the sensor was not sensitive enough to determine the concentrations below that range. We proposed to apply anti-fibrinogen antibodies in building the sensors to increase the sensitivity especially for the detection of ultra-low levels of fibrinogen. It will certainly broaden the detection range of fibrinogen and make the application in disease screening and health monitoring possible.
CHAPTER 5
DNA FUNCTIONALIZED SWNT GAS SENSOR ARRAY FOR BREATH ANALYSIS

The possibility of routine monitoring of metabolic disorders via breath analysis has attracted considerable scientific and clinical interests for many years. The volatile organic compounds (VOCs) in exhaled breath, which are mainly blood borne, particularly provide valuable information about the subject’s physiological and pathophysiological conditions. Additionally, it is non-invasive, real-time, painless and agreeable to patients. We have developed a wireless sensor array based on ssDNA-decorated SWNT for the detection of some physiological indicators in breath. Four DNA sequences were used to functionalize SWNT sensors to detect trace amount of methanol, benzene, acetonitrile, dimethyl sulfide, hydrogen sulfide, and acetone, which are indicators of heavy smoking, excessive drinking, and diseases such as lung cancer and diabetes. Our tests indicated that DNA functionalized SWNT sensors exhibit great selectivity, sensitivity, reproducibility, and repeatability. Furthermore, different molecules can be distinguished through
pattern recognition enabled by this sensor array. Thus, this sensor array has demonstrated a very high potential to be applied in chemical or bimolecular detection for disease diagnostics and health monitoring.

5.1 INTRODUCTION

Breath analysis starts from the early history of medicine, when ancient physicians knew that the odor of a patient’s breath can be associated with some diseases. Breath was also believed to provide insight into the physiological and pathophysiological processes in patients’ bodies, e.g. the sweet smell of acetone accompanies diabetes [116-118] (Fig. 5.1). Modern breath analysis began in the 1970s when Pauling detected around 200 different VOCs in exhaled breath by gas chromatography [226]. Breath analysis, as a diagnostic technique, is non-invasive, painless, agreeable to patients, achievable in real time, and can even provide information beyond the conventional analysis of blood and urine [119-120, 122, 227-229]. A correlation between the concentration patterns of the VOCs in breath and the occurrence of certain diseases was demonstrated [122].
Many different analytical techniques were used to analyze exhaled breath, such as gas chromatography and mass spectrometry (GC and MS) [121-122, 226, 230-240]. However, they require laboratory setting, significant processing time, very costly reagents, expensive instrumentation, and highly trained professionals. Consequently, it cannot be used for individual health monitoring at home or other daily activities. Our goal is to develop a portable, real-time, accurate, easy to use, and cost effective device for breath analysis.
SWNT, with their specific electrical, mechanical, optical, chemical, and thermal properties, are widely utilized in chemical/biological sensors [17], electrical interconnects [18], agents for drug delivery [21-22], low voltage, cold-cathode field-emission displays [23] and nanoscale circuits for beyond silicon based complementary metal-oxide-semiconductor (CMOS) electronics [24-26].

DNA decorated carbon nanotubes are novel nanoscaled materials that consist of SWNT coated with a self-assembled monolayer of ssDNA. This unique system offers an intriguing combination of properties of an essential and ubiquitous biomolecule-DNA and one of the most heralded inorganic nanomaterials-SWNT. It has integrated the selective odorant interactions of ssDNA [89] with the sensitivity of SWNTs to the changes of its surface electronic environment when exposed to analytes [90]. Moreover, the response of these devices to a particular chemical of interest can always be optimized by changing the base sequence of the ssDNA. Functionalization of SWNTs with DNA has demonstrated attractive prospects in various fields, including detection of chemical vapors, solubilization in aqueous media, and nucleic acid sensing [38, 91-92].

Exhaled breath mainly consists of oxygen, nitrogen, carbon dioxide, water, and inert gases. The trace components, more than 200 different VOCs (ppb level), make up the rest of breath. In order to recognize certain molecules in breath, a sensor array of different DNA-decorated SWNT sensors is created and different chemicals are distinguished from each other through pattern recognition.

Here we introduce a wireless sensor array with six channels to measure the responses of six DNA-SWNT sensors simultaneously when exposed to different gases, which can certainly reduce the undesirable noises of SWNT to interference analytes. Ultrathin films of SWNT were assembled onto the microelectrodes by a low temperature and low cost Dielectrophoretic (DEP) assembly process. Then ssDNA of different sequences were noncovalently bonded to the SWNT surfaces, enhancing their responses to the gas vapors. Various DNA decorated SWNT sensors respond differently to different gases. Thus, this real-time wireless sensor array generates one specific pattern for one particular gas, and it can be utilized in turn to recognize certain chemicals.
Seven chemicals were selected: (1) water, the common component in breath, (2) methanol, a possible indicator for excessive drinking [241-242], (3) benzene, a marker that at high levels related with heavy smoking [230-231, 243], (4) acetonitrile, which is also related with smoking [230, 244-246], (5) dimethyl sulfide, a potential indicator for lung cancer [247-251], (6) hydrogen sulfide, a probable indicator of bad breath, and (7) acetone, an acknowledged biomarker for diabetes [116-118, 252].

5.2 EXPERIMENTAL PROCEDURES

The sensing system includes the DNA functionalized SWNT sensor array and a wireless sensing package. We first tested our sensing system with methanol vapor, and then detected seven chemicals which are very important indicators for general health.

5.2.1 FABRICATION OF SS-DNA DECORATED SWNT SENSOR ARRAY

First, microelectrodes with 3 μm gap were fabricated by photolithography followed by sputtering Cr/Au (20 nm/150 nm) layer onto a silicon oxide substrate, while aluminum was deposited on the top of SiO₂ layer as the sacrificial layer before photolithography (Fig. 5.2 (1-4)). Then by the solution-based DEP assembly which is a low temperature, low cost, but very efficient method for placing nanotubes, SWNTs were assembled between the microelectrodes, just as bridges (Fig. 5.2 (5)). Fig. 5.3 shows nanotubes assembled at one microelectrode. In this assembly, a large number of individual nanotubes bridged the microelectrodes. Majority of the nanotubes assembled were reasonably aligned along the gap between the two microelectrodes, while there were few mis-aligned nanotubes. The few misalignments may be caused by the sudden remove of the electrical field. Some nanotubes didn't have enough time to be well aligned between the two electrodes when the electrical field was put off. The SWNTs used here (diameter: 1~2 nm; length: 2~5 μm) were purchased from Brewer Science Company. They were then dispersed in DI water to reach the final concentration of 4g/L. An AC (Alternating Current) signal of 1V pp and 10MHz
frequency was applied between the electrodes after the placement of 2 μL of the dispersed SWNTs solution onto the top of the electrode gap. After aligning SWNTs between the microelectrodes, ssDNAs with different sequences were utilized to functionalize SWNTs (Fig. 5.2 (6)). The chosen DNA sequences in this study are shown below:

DNA 24GT: GTGTGTGTGTGTGTGTGTGTGTGT
DNA 24A: AAAAAAAAAAAAAAAAAAAAAAAAA
DNA 24Aa: Amine-AAAAAAAAAAAAAAAAAAAAAAAAAA-Amine
DNA 24Ma: Amine-GACCTGTCAAGGACCTGTCTTCAAG-Amine
DNA 24G:     GGGGGGGGGGGGGGGGGGGGGGG
DNA 32G:     GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG

Fig. 5.2. Fabrication procedure for DNA decorated SWNTs on microelectrodes.

As mentioned earlier, DNA can be self-assembled on SWNTs by noncovalent bonding via π-π stacking force. This can be easily achieved by dropping 5 μL of 100 μM ssDNA solution onto the SWNTs and incubating in a chamber with 100% humidity for 1 hour. The extra DNA solution was removed by compressed air. This fabrication procedure is schematically exhibited by Fig. 5.2.
Fig. 5.3. SEM photograph of SWNTs assembled on the microelectrode.

During the DEP process, the SWNTs were aligned between the two electrodes by the applied AC electrical field. Dielectrophoresis is a phenomenon in which a force is exerted on a dielectric particle when it is subjected to a non-uniform electric field (eq 5.1). The strength of the force depends strongly on the medium and the particles' electrical properties, on the particles' shape and size, as well as on the frequency of the electric field, which can be certified by the formula:

\[
F_{\text{DEP}} = \frac{\pi r^2 l}{3} \varepsilon_m \text{Re} \left\{ \frac{\varepsilon_p - \varepsilon_m}{\varepsilon_m} \right\} \nabla \left| \frac{E}{E} \right|^2
\] (5.1)

Besides voltage, the amount of assembled SWNT can also be determined by the applied frequency, current, shape of the electrodes, duration time, the particles' shape and size and the concentration of carbon nanotubes. The optimum amount of SWNT is determined by the best sensing performance. Too much SWNT been assembled on the electrode will decrease the contact area of nanotubes to gas molecules, thus will lower the resolution; while too little SWNT will increase the signal's instability. The amount of assembled SWNT is represented by the resistance between the two electrodes, and the best sensing performance is provided by the SWNT with a resistance ranging from 5 to 15 kΩ.

Although there are some other approaches to position nanotubes onto electrodes, such as self-assembly [253], random spreading [254], direct growth [255], and nanomanipulation [256], each of
these techniques has its limitations, e.g., high temperature of 900 °C [255]. The DEP assembly has overcome these limitations and is a versatile and flexible way to assemble carbon nanotubes onto CMOS circuitry. It is a simple and powerful method to manipulate and trap micro-and nanoparticles [257]. As a CMOS compatible method, DEP assembly can quickly be utilized to incorporate carbon nanotubes onto platforms as components of microsystems or for in-line characterization or as nanostructure after manufacturing.

SWNTs bundles were successfully aligned between the microelectrodes (Fig. 5.4). The observed tiny white dots, which were believed to be aggregated ssDNA molecules (according to one previous report [258]), were randomly dispersed in the SWNTs bundles. These decorated DNA molecules have almost spread everywhere in the SWNTs bundles. ssDNA was coated onto the SWNT by wrapping on the surface wall of SWNTs, which relied on the π-π stacking of nitrogenous bases of DNA on the aromatic rings of SWNT. The reason why we could not see the increase in the thickness of the SWNT bundles is that the SEM imaging was not sensitive enough to resolve such difference. It has been reported by Staii et. al. that ssDNA formed a ~1 nm layer on SWNTs [38]. However, the observation of DNA aggregation, displayed as the tiny white dots, is consistent with the AFM results reported by Jeng et. al., where DNA was adsorbed and folded on the surfaces of the nanotubes in a non-uniform manner [258].
5.2.2 WIRELESS SENSING PACKAGE

The functional block diagram of our wireless nanosensor array is shown in Fig. 5.5a. This design is based on the modular design concept and has been developed further. The major advantage of this module design method is that it provides a flexible and universal platform to approach a wide variety of applications. For different sensors, the signal conditioning module can always be reprogrammed and adjusted, allowing diverse sensors to be integrated in this wireless sensing platform.

The actual system developed based on this modular concept is shown in Fig. 5.5b. In the figure, the nanosensor array module is on the top layer, the signal conditioner is on the middle one, while the bottom layer contains low power microcontroller board called Waspmote board and a RF module.

The Waspmote board contains an in-built accelerometer and when associated with the GPS module, can be utilized to measure real-time speed, direction and location of the sensor. This board also possesses an outstanding power management which allows it to be used in some remote places or under adverse conditions, and an optional solar panel can permit almost any indefinite operation. In addition, the RF module can be chosen for a different mode with various protocols such as
Bluetooth, ZigBee, IEEE802.15.4 and standard RF depending on the wireless range of certain given applications and the need for bidirectional communications which varies from 30m to 10 km. Furthermore, the usage of flash memory tool enables the remote nodes to acquire data on command from a base station, or by an incident sensed by one or more inputs to the node. The major specifications are listed in Table 5.1.

Table 5.1. Specifications of the system

<table>
<thead>
<tr>
<th>Microcontroller:</th>
<th>ATmega1281</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency:</td>
<td>8 MHz</td>
</tr>
<tr>
<td>Temperature:</td>
<td>[-20 ºC, +65 ºC]</td>
</tr>
<tr>
<td>Clock:</td>
<td>RTC (32 KHz)</td>
</tr>
<tr>
<td>Power (ON):</td>
<td>(3.3 - 4.2) V X 9 mA</td>
</tr>
<tr>
<td>Input/Output:</td>
<td>7 Analog (I), 8 Digital (I/O), 1 PWM, 2 UART, 1 I2C, 1 USB</td>
</tr>
</tbody>
</table>

5.2.3 SENSING STRATEGIES AND CHARACTERISTICS

The collected data with above nanosensor array are wirelessly transmitted to a Wasmote gateway which is attached to the PC via a USB port. A graphic user interface (GUI) program developed by LabWindows/CVI was designed to enable customer applications after collecting the data from Wasmote gateway. Fig. 5.6 shows the GUI interface for this purpose. The six windows show the responses of six different sensors concurrently when exposed to analytes vapor where the x-axis represents the obtained data sequence at sampling rate of 2 Hz and the y-axis is the resistance of the nanosensor. The reason of this sequence dependent sensing response could be due to the amount of SWNTs assembled and the different conformation of DNA decorated on SWNT. In our application, data acquired was real-time monitored, plotted simultaneously and stored for further analysis.
5.2.4 SENSOR CHARACTERISTICS AND METHANOL DETECTION

Methanol is a clear, colorless liquid that looks like water and has no discernible odor in low concentration. It is used primarily for the manufacture of other chemicals and as a solvent, but acute exposure to methanol is very harmful to human. In one study, symptoms of blurred vision, headaches, dizziness, nausea and skin problems were reported in teacher aides exposed to duplicating fluid containing 99% methanol [259]. The concentration in the breathing zones near the machines in twelve schools ranged from 485 to 4096 mg/m³ (365 to 3080 ppm) for a 15 minute sample.

It is reported that the binding affinities between ssDNA and SWNT follow the trend \( d(G)_{21} - \text{SWNT} > d(A)_{21} - \text{SWNT} > d(C)_{21} - \text{SWNT} > d(T)_{21} - \text{SWNT} \) [260], and from earlier work in our group, the DNA length of 24 is the optimum DNA sequence length for methanol sensing [261]. Thus, DNA 24G- and DNA 32G-decorated SWNT sensors were chosen to test the effectiveness of this
sensing system. We built the sensor array with two bare SWNT sensors, two DNA 24G-functionalized SWNT sensors, and the other two DNA 32G-functionalized SWNT sensors, and the responses when exposed to methanol vapor were collected and compared.

Fig. 5.7. Normalized resistances of DNA 24G, DNA 32G-functionalized SWNT nanosensors and of bare SWNT when exposed to methanol vapor.

The sensor array was applied to detect methanol vapor (50 ppm) at room temperature for sensor characterization. It was mounted onto a testing board with the signal processing unit and a wireless transmitter (Fig. 5.5b). This system functions as an autonomous unit for gas monitoring. Then the board and the petri dish containing methanol were confined in a sealed chamber (Fig. 5.8). Changes in resistance, which can reveal the presence of certain chemicals, were recorded and displayed on the GUI interface (Fig. 5.6).
The sensing part was exposed to air, methanol vapor, and air consecutively for three times, and the normalized resistances of the six sensors were collected and plotted in Fig. 5.7. It is reported that the resistance of SWNT sensors remained almost the same when they were exposed to the ambient air until certain specific gases became the subjects [94, 260]. Our group's former research also confirmed this point [95]. The bare SWNT sensors had an instant response to the methanol vapors and could almost return to their initial resistances in air. But, functionalizing the SWNT sensor with DNA 24G and DNA 32G improved the performance of bare SWNT sensors by 300%, and 200%, respectively (Fig. 5.9). For SWNT sensors functionalized with the same DNA sequence, the changes in resistance for methanol were almost the same. This confirmed the sensing characteristics of DNA decorated SWNTs with two independent sensors. For SWNTs functionalized with different DNA sequences, the results demonstrated that odor responses of DNA-decorated SWNT sensors are sequence dependent.

Fig. 5.9. Resistance changes of DNA 24G, DNA 32G-functionalized SWNT nanosensors and of bare SWNT sensors when exposed to methanol vapor.

5.3 RESULTS AND DISCUSSION
Breath analysis targets - water, methanol, acetone, acetonitrile, benzene and DMS vapors were generated from the solutions in a petridish placed in a sealed chamber at room temperature. Since acetone and DMS are extremely volatile, DPG was utilized to dilute the acetone (6 µL acetone dissolved in 50 mL DPG) and DMS (5 µl DMS dissolved in 35 ml DPG solution) solutions. Nanotubes do not respond to DPG, therefore, the change in resistance will be only caused by DMS [262]. The equilibrium vapor pressures of water, methanol, benzene, acetonitrile, acetone and DMS at 20°C are 18.66, 97.66, 70, 71, 0.038 and 0.076 torr respectively. Hydrogen sulfide was generated from the reaction between FeS and H₂SO₄. The vapor pressure inside the chamber after complete reaction is estimated to be 0.027 torr (36 ppm). DNA 24GT, DNA 24A, DNA 24Aa, and DNA 24Ma were decorated on SWNT sensors and their responses to these vapors were measured.

In this assembly, a large number of individual nanotubes bridged the microelectrodes. The majority of the nanotubes assembled were successfully aligned between the two microelectrodes (Fig. 5.10a). The tiny white dots, believed to be aggregated ssDNA molecules, were coated onto the SWNT bundles through π-π stacking.

![SEM image of ssDNA-decorated SWNTs assembled between microelectrodes](image)

![Resistance changes of DNA 24GT, DNA 24A, DNA 24Aa and DNA 24Ma-functionalized SWNT nanosensors and of bare SWNT when exposed to water, methanol, benzene, acetonitrile, dimethyl sulfide, hydrogen sulfide and acetone vapors. Error bars = ± standard deviation and n = 5.](image)
Five different nanosensors decorated with the same DNA sequence were used to detect each chemical. The resistance changes after exposure to the chemical vapors for 10 minutes were recorded (Fig. 5.10b).

Water, methanol, acetone, and acetonitrile are polar molecules and hydrophilic. Benzene is a nonpolar organic molecule with very limited solubility in water (hydrophobic). Hydrogen sulfide and dimethyl sulfide (DMS) are polar molecules but hydrophobic. For methanol and water, which are polar molecules with similar structure, the responses had the same trend (the red and black curves in Fig. 5.10b). However, since methanol is more volatile and has a higher vapor pressure at room temperature, the resistance changes for methanol (the red curve) is higher than that for water (the black curve). The sensor array’s response to acetonitrile was similar to methanol, since acetonitrile was also a polar molecule and hydrophilic, but was much smaller compared to that for methanol. It is because the nitrile group (C≡N) is less hydrophilic than the hydroxyl group (O-H). Therefore, the affinity of DNA to acetonitrile is less strong than it to methanol. For acetone, a hydrophilic polar molecule, the pattern of response was similar to water and methanol’s, but the resistance changes were much smaller resulting from a lower polarity and weaker hydrophilic property due to the carboxyl group (C=O) and two methyl groups. DNA decorated nanosensors barely responded to benzene and DMS (the green and blue curves). It is because benzene and DMS are hydrophobic molecules which do not tend to adsorb on the DNA decorated SWNTs. Especially for benzene, DNA decorated SWNT sensors exhibited even smaller responses compared to bare SWNT sensors. For hydrogen sulfide, the response pattern was different from all the others. The resistance of SWNT sensor decorated with DNA 24GT decreased significantly when exposed to hydrogen sulfide. However, the resistances of the other nanosensors all slightly increased when exposed to hydrogen sulfide. It is very likely that the interaction between nucleobases G and/or T with free thiol group (-SH) is much stronger than that of nucleobase A and C. It can be due to the highly polarizable divalent sulfur centers in hydrogen sulfide. This unique response of the DNA 24GT decorated SWNT sensor to hydrogen sulfide can be used to differentiate it from other vapors.
Study of the concentration and temperature effects is in progress and will better demonstrate our sensor array’s high selectivity and sensitivity.

5.4 CONCLUSION

We have developed a wireless nanosensor array based on ssDNA decorated SWNT on micro devices. The DNA functionalized SWNT sensors presented reversible and repeatable changes in response to different vapors. The nanosensor array, decorated with four different DNA sequences, was tested with seven vapors which can indicate the physiological and pathophysiological conditions of individuals. The results indicated that DNA increased the affinity of SWNTs to hydrophilic molecules, which was due to the surface properties of SWNTs being altered from hydrophobic to hydrophilic by the DNA decoration. In addition, the DNA 24GT decorated SWNT sensor exhibited a different behavior (decrease in its resistance) compared to other types of SWNT sensors when exposed to hydrogen sulfide. This showed a great potential to distinguish hydrogen sulfide from other vapors by DNA 24GT decorated SWNT sensor. Additionally, measuring the responses from six different DNA functionalized SWNT sensors simultaneously and analyzing the response pattern will allow one to selectively detect different analytes. This array-based sensing approach provides high selectivity, good sensitivity, great repeatability and excellent precision for gas analysis. It reveals high potential for real-time and highly sensitive breath analysis for non-invasive disease diagnostics and health monitoring.
CHAPTER 6

DNA FUNCTIONALIZED SWNT GAS SENSOR ARRAY - THE EFFECT OF DNA SEQUENCE AND LENGTH

Nine ssDNA sequences were used to functionalize SWNT sensors to determine the effect of DNA sequence and length by detecting the trace amount of acetone and HCl in vapor. DNA 24Ma (24 randomly arranged nitrogenous bases with one amine at each end of it) decorated SWNT sensor and DNA 24A (only adenine (A) base with a length of 24) decorated SWNT sensor have demonstrated the largest sensing responses towards acetone and HCl, respectively. On the other hand, for the DNA GT (base pair of guanine and thymine) decorated SWNT sensors with different sequence lengths, the optimum DNA sequence length for acetone and HCl sensing is 32 and 8, respectively. Thus, it is very obvious that the sensing performance of DNA functionalized SWNT sensors is highly sequence dependent. The selection of optimal DNA sequences for the detection
of certain breath biomarkers is therefore crucial in order to use this sensor array for noninvasive
disease diagnosis and monitoring through breath analysis.

6.1 INTRODUCTION

SWNTs are widely used in sensing applications due to their desirable properties such as fast
response, high sensitivity and miniature size. However, the nonspecificity of SWNTs reacting to
various gases and vapors renders it hard to apply plain SWNTs in the detection of certain chemicals.
Therefore, surface chemistry is crucial to tailor the properties of SWNTs and to achieve desired
sensitivity and selectivity to targeted analytes. DNA has previously been decorated on SWNT to
enhance their specificity and sensitivity in gas sensing and biosensing as well. While a large amount
of results have demonstrated that specific detection of chemicals/biomolecules using ssDNA-
SWNT sensors is a much improved alternative to plain SWNT sensors, the understanding of the
sequence dependent sensing mechanism still needs further investigation.

The impact of DNA sequence and oligonucleotide length has been studied in fluorescent
DNA biosensing [263], barcoding of fungi [264], as well as in gas sensing [265]; here we want to
focus on this effect on chemical sensing. We emphatically studied and compared the detection of
acetone vapor and hydrogen chloride gas using our DNA-SWNT sensor array. The seven DNA
sequences utilized in the detection of acetone and HCl are 24A, 24Aa, 24GT, 24Ma, 8GT, 16GT,
and 32GT. The reason that we selected DNA sequences of 24A, 24Aa was to examine the effect of
amine group for the sensing performance of such DNA sensors, while DNA 24Ma (a mixed
sequence with two amine groups at the two ends) could clarify whether increasing the diversity of
the DNA bases will improve the sensing results. On the other hand, wrapping of CNTs by ssDNA
was found to be sequence-dependent. DNA-GT series can readily wrap around SWNTs based on
the electrostatics of the DNA-CNT hybrid. Additionally the d(GT)n-CNT hybrids have a much
more uniform periodic structure [266], which makes them a great candidate to study the length
dependence upon the gas sensing of acetone and HCl.

The reason we selected acetone is because it is commonly used in chemical reagent industry,
which can cause illnesses to human bodies. As it evaporates rapidly and undoubtedly hazardous to
human healthy, it is required to be monitored. According to the Environmental Fact Sheet from
New Hampshire Department of Environment Service, irritation of the eyes and respiratory system,
mood swings, and nausea are seen in humans exposed to Acetone with the concentration of 500
ppm in air and greater. It was reported that individuals may develop headache, tiredness, dizziness,
allergy, and even unconscionessence when exposed to the air which contained high acetone vapor
concentration [267]. Thus, it is necessary to detect and monitor the level of acetone vapor
concentration in the environment for our safety. On the other hand, acetone vapor can also be
utilized as a marker for some disease diagnostics. It is reported that acetone concentration in the
breath of a healthy person is around 5 ppm, while the level of acetone for a patient with diabetes
mellitus reaches 300 ppm. Thus, acetone can be a breath marker for diagnosis of diabetes [252].

Besides volatile organic compounds, during the last few decades, the emission of pollutant
gases, such as HCl, SO₂, SO₃, CO, NO, NO₂, and CO₂, has become a serious problem. HCl is one
of the major contributors to the acid rain as it can easily dissolve in water and become a strong
corrosive acid. HCl gas is mainly generated from burning fuels which contain chlorine like coal,
and incinerating waste which includes plastic. The review reports that the coal burning plants
produce 14-220 ppm HCl gas while incinerators show 215-1250 ppm of HCl gas emission [143].
HCl, is a pulmonary irritant with intermediate water solubility that can cause acute damage in the
upper and lower respiratory tract. It is also highly corrosive to metals and skins. Many countries
have introduced some strict regulations to control and reduce the emission level of HCl gas, since
the quality of environment is directly related to the health of humans and wildlife. Thus, monitoring
of the HCl gas is urgently needed.
6.2 EXPERIMENTAL PROCEDURES

The fabrication procedures are the same as described in Chapter 5 (Fig. 5.2). First, microelectrodes with a 3 μm gap were fabricated by photolithography followed by E-beam depositing a Cr/Au (20 nm/150 nm) layer onto a silicon oxide substrate. Then SWNT (diameter: 1~2 nm; length: 2~5 μm) were assembled between the microelectrodes, just as bridges via solution-based DEP assembly. An AC signal of 1Vpp and 10MHz frequency was applied between the electrodes after the placement of 2 μL of the dispersed SWNT solution onto the top of the electrode gap. After aligning SWNT between the microelectrodes, DNA were assembled on SWNT through π-π stacking. DNA sequences used to study the effect of their sequence and length in gas sensing performance are shown below:

In terms of sequence:

DNA 24A : AAAAAAAAAAAAAAAAAAA
DNA 24Aa: amine-AAAAAAAAAAAAAAAAAAAAAAAAAAAA-amine
DNA 24GT: GTGTGTGTGTGTGTGTGTGTGT
DNA 24Ma: amine-GTCTTACGCTAGCTGGGCATTACG-amine

In terms of length:

DNA 8GT : GTGTGTGT
DNA 16GT: GTGTGTGTGTGTGTGT
DNA 24GT: GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT
DNA 32GT: GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT
Acetone is a very volatile organic compound, thus it is very easy to obtain acetone vapor from acetone solution. In order to obtain 50 ppm acetone vapor, we used DPG to adjust the acetone partial pressure in atmosphere. DPG is a mixture of three isomeric chemical compounds: 4-oxa-2, 6-heptandiol, and 2-(2-Hydroxy-1-methyl-ethoxy)-propan-1-ol. It is a colorless, nearly odorless liquid with a high boiling point and low toxicity. Due to its extremely low vapor pressure (0.06 mm Hg @ 25 °C), DPG has been widely employed to modify the partial pressure of analytes. In addition, SWNTs have no response to DPG, thus the responses of SWNT sensor to acetone solution diluted with DPG is purely caused by acetone [95]. The 50 ppm acetone vapor was generated by mixing 6 μL acetone solution with 50 mL DPG. The vapor pressure of acetone at room temperature is about 184 torr (according to Sigma-Aldrich), while that of DPG at 20 °C is less than 0.01 torr (from Sigma-Aldrich), which is extraordinary smaller than that of acetone.

The sensor array was exposed to air, acetone vapor, and air consecutively for two cycles, and the normalized resistances of four sensors were collected and plotted in Fig. 6.1. The resistances of the sensors when exposed to air almost plateaued. Therefore they can be used to normalize the resistances of the sensors when exposed to acetone. Each exposure time was about 20 minutes, and

\[
\chi_{\text{acetone}} = \frac{n_{\text{acetone}}}{n_{\text{acetone}} + n_{\text{DPG}}} = \frac{0.791 \times 6 \times 10^{-3}}{1023 \times 50 + 0.791 \times 6 \times 10^{-3}} = 2.15 \times 10^{-4} \quad (6.2)
\]

Here, \(\chi_{\text{acetone}}\) is the mole fraction of acetone (eq 6.2), and \(P_{\text{acetone}}\) is the vapor pressure of pure acetone solution. Since the vapor pressure of acetone at room temperature is about 184 torr, the vapor pressure generated by this diluted acetone solution is 0.03956 torr (eq 6.1). Divided by 760 torr atmospheric pressure, the concentration of acetone vapor in air is around 50 ppm. The acetone in DPG solution was put into one petri-dish and was exposed to the sensor array in a sealed chamber during the measurements. When the chamber was opened, the sensor array was exposed to ambient air, the resistance of the six sensors all dropped simultaneously (Fig. 6.1).

\[
\text{Vapor pressure of acetone in DPG solution is } P_{\text{acetone}} = \chi_{\text{acetone}} \times P_{\text{acetone}} \quad (6.1)
\]
each time the acetone vapor was replaced by air, the sensor could almost regenerate. The reason for continued increase in the resistance over time, when exposed to acetone for 20 minutes is probably because the acetone molecule is much bigger than methanol one, so the adsorption of acetone onto the sensor is harder, taking more time to reach an ultimately stable stage. The sensor made by Salgado et al.'s group also displayed instability towards detecting acetone [268]. Moreover, it's reported that the sensitivities vary at different operating temperatures and the sensor is more sensitive to methanol than acetone until reaching very high temperature (above 325 °C) [269]. The time it takes acetone to get saturated in the air is also temperature dependent [270]. But as long as the resistance can sustain itself in air, the resistance changes when exposed to subject gases can reliably be used for detection of certain gases. The two cycles of testing confirmed the repeatability and reversibility of the nanosensors to acetone.

![Fig. 6.1. Normalized resistances of DNA 8GT (a), DNA 32GT (b), DNA 24Aa (c) - functionalized SWNT nanosensors and of bare SWNT (d) when exposed to acetone.](image)
Compared to the responses of other DNA sequences with the same length-24 bases (Fig. 6.2a), the DNA Ma-functionalized SWNT nanosensor exhibited the highest resistance change (10.15%), and it is much larger than the response of bare SWNT sensor (3.03%). The DNA A, DNA GT, and DNA Aa-functionalized ones showed resistance changes in a decreasing order. The responses of DNA GT with different sequence lengths when exposed to acetone were compared in Fig. 6.2b. The changes in resistance of SWNTs functionalized with DNA of different sequence lengths varies. The DNA 32GT functionalized SWNT sensor gave the best performance, followed by DNA 8GT, DNA 16GT and DNA 24GT. All DNA decorated SWNT sensors demonstrated larger changes in resistance towards acetone vapor, which indicated that the DNA modification had greatly improved the sensitivity of bare SWNT sensors. For the reproducibility, revealed by the variation of $\Delta R/R_0$ among different sensors of the same type, bare SWNT sensors have the smallest value (0.138); while the DNA functionalized SWNT sensors show slightly bigger deviations (0.277~1.125) (Table 6.1). The variation of the sensing performance between different DNA functionalized SWNT sensors is because the decoration conditions cannot be the same, and the affecting parameters can be assembly temperature, moisture level, operations, etc.

Fig. 6.2. Resistance changes of DNA 24GT, DNA 24A, DNA 24Aa, DNA 24Ma (a), and DNA 8GT, DNA 16GT, DNA 24GT, DNA 32GT (b)-functionalized SWNT sensors and of bare SWNT nanosensors when exposed to acetone vapor.
Table 6.1. Responses of DNA decorated SWNT sensors to acetone at room temperature.

<table>
<thead>
<tr>
<th></th>
<th>SWNT</th>
<th>DNA 24A</th>
<th>DNA 24Ma</th>
<th>DNA 8GT</th>
<th>DNA 16GT</th>
<th>DNA 24GT</th>
<th>DNA 32GT</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔR/R₀ (%)</td>
<td>3.03±0.138</td>
<td>8.88±0.466</td>
<td>5.82±0.338</td>
<td>10.15±0.667</td>
<td>9.85±1.04</td>
<td>6.71±0.844</td>
<td>8.23±0.277</td>
</tr>
</tbody>
</table>

6.3.2 HYDROGEN CHLORIDE DETECTION BY SENSOR ARRAY

Unlike acetone which can simply be generated from its evaporation from a solution, HCl vapor generated from 37% HCl solution will contain water vapor which can significantly influence the sensing response of DNA-SWNT sensors. We therefore utilized a chemical reaction between NaCl and concentrated H₂SO₄ solution to generate HCl gas. The concentrated H₂SO₄ solution will not generate vapor, thus it will not interfere with the HCl gas testing, and it can also act as a dehydrate agent. Using the same test setup, we placed concentrated H₂SO₄ into the petri dish at first, and then added 0.5mg NaCl. Due to the heat release from the chemical reaction, we wait for 1 minute. Then the sensor array was put into the chamber with generated HCl gas. The concentration of HCl gas in the chamber was estimated to be 50 ppm.

Unlike the positive change of resistance when the nanosensors were exposed to acetone vapor, they exhibited a decrease in resistance when exposed to HCl gas (Fig. 6.3). Besides, the reduced resistance of most nanosensors cannot recover to the initial value when the HCl gas is switched back to air. For SWNTs decorated with DNA 24A and DNA 24Ma, the resistances returned to their initial values when HCl gas was replaced by air, while the nanosensors functionalized with other DNA sequences and the bare SWNT sensor could hardly regenerate. The different responses may result from the different affinities between SWNTs and DNAs of different sequences. Such drift of the baseline, represented by the resistance in ambient air, is possibly caused by the intercalation of the SWNT bundles with HCl molecules and the interaction of HCl with adventitious impurities. The interaction of HCl with adventitious impurities may change the intertube contact resistance. Some of the binding sites were irreversible, and once they were bonded to nanotubes, they were
very hard to get desorbed, which made the sensor unable to regenerate in an ambient environment. The incomplete recovering of SWNTs after testing with HCl gas was consistent with the results reported by other groups [271]. In order to solve this problem, several methods have been reported to recover the sensing ability of SWNT sensors. For instance, Bekyarova et al functionalized the SWNT with different organic chemicals and facilitated the regeneration of SWNT sensor to HCl gas [271]. They used covalent bonding to decorate different organic chemicals onto the single-walled carbon nanotube to attract HCl. The presence of these functional groups allows the indirect attachment of the HCl molecules on SWNTs as the functional groups will contact the HCl molecules before HCl molecules get the chance to attach on SWNTs. In this case, the HCl will not affect the properties of the SWNT bundles. The best recovery performance was introduced by the group-COOC₅H₄N, as its conjugate acid might have the necessary oxidation potential to enable the electron transfer from the semiconducting SWNTs. It resulted in introducing holes into the valence band and decreasing the resistance. A similar mechanism has been reported and verified [272-273]. There are some other methods for the SWNT sensors to regenerate, including heating the device to high temperature [8] and UV irradiation [32].
As to our functionalization, the DNA molecules were non-covalently bound to the SWNT, which may not be as strong as covalent bonding. And more importantly, DNA molecules cannot provide holes into the valence band to allow electron transfer from SWNTs, while the organic functional groups used in Bekyarova et al.'s study can. So, perhaps we can improve the sensor's regeneration by functionalizing SWNT with some organic functional groups before decorating DNA onto it as long as the organic functional group won’t affect the functions of DNA. Even
without rectification of the sensor’s recovery condition, we can still detect trace amount of HCl gas based on the data collected with the sensor array.

Table 6.2. Responses of DNA decorated SWNT sensors to HCl at room temperature.

<table>
<thead>
<tr>
<th>HCl</th>
<th>SWNT</th>
<th>DNA 24A</th>
<th>DNA 24Aa</th>
<th>DNA 24Ma</th>
<th>DNA 24GT</th>
<th>DNA 16GT</th>
<th>DNA 24GT</th>
<th>DNA 32GT</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔR/R₀ (%)</td>
<td>-8.56±1.170</td>
<td>-21.92±1.257</td>
<td>-17.01±1.299</td>
<td>-14.75±1.009</td>
<td>-19.62±0.972</td>
<td>-12.31±1.006</td>
<td>-8.53±0.650</td>
<td>-11.82±0.776</td>
</tr>
</tbody>
</table>

Comparing the different DNA sequences with the same length-24 bases (Fig. 6.4a), DNA 24A-functionalized SWNT nanosensor shows the highest resistance decrease (21.92%), which is much larger than the value of the bare SWNT sensor (8.56%). The DNA decorated SWNT sensors react with HCl gas in the order of DNA 24A > DNA 24Aa > DNA 24Ma > DNA 24GT. DNA 24GT gave even a little less resistance change than bare SWNT (Fig. 6.4a), which means it barely improved the performance of the sensing of HCl gas. For DNA GT composed of different sequence lengths, the responses of DNA-SWNT sensors were also different (Fig. 6.4b). As to HCl gas, the DNA 8GT functionalized SWNT nanosensor gave the best performance, while DNA 16GT and DNA 32GT were almost the same, ranking second.

Fig. 6.4. Resistance changes of DNA 24GT, DNA 24A, DNA 24Aa, DNA 24Ma (a), and DNA 8GT, DNA 16GT, DNA 24GT, DNA 32GT (b)-functionalized SWNT nanosensors and of bare SWNT when exposed to HCl gas.

6.3.3. SENSOR SENSING PERFORMANCE
The detection results of DNA-functionalized carbon nanotube sensors are highly repeatable for different sensors; no matter the analyte is acetone vapor (Fig. 6.5a and b) or HCl gas (Fig. 6.5c and d). The device to device variation is around 5%, so the DNA modified SWNT sensors provide particular high accuracy.

Fig. 6.5. Repeatability with different sensors on one array. a, b) Normalized resistances of different DNA 16GT-functionalized SWNT nanosensors when exposed to acetone; c, d) Normalized resistances of DNA 8GT-functionalized SWNT nanosensors when exposed to HCl gas.

It is clearly shown that one specific SWNT nanosensor, regardless of whether it was with functional groups or not, demonstrates different responses to different analytes (Fig. 6.6). The nature of ssDNA-SWNT interactions plays an indispensable role in the sensing performance of the DNA-SWNT sensors. Stronger binding sites permit greater amount of ssDNA to be bonded onto the SWNT surfaces, which facilitates the adsorption of polar analytes [260], and this may explain the specificity of the DNA sequence used to decorate the device on the performance in gas sensing.
Normally, functionalized SWNT nanosensors reveal better sensitivity than bare SWNT ones. From the results of seven different DNAs functionalized SWNT sensors, it is very likely the DNA 24Ma and DNA 32GT can be used to sense acetone vapor, while the DNA 24A and DNA 8GT can be employed to detect HCl gas. However, with our sensor array, we can simply use the pattern generated by the different sensors in just one array to distinguish different analytes (Fig. 6.6). Such pattern recognition is applicable to the situation when all gas subjects are present at the same concentration.

![Graphs showing sensing responses to acetone vapor and HCl gas.](image)

Fig. 6.6. Comparison of the sensing responses to acetone vapor and to HCl gas.

### 6.4. CONCLUSION

It is obvious that the sensing performance of DNA functionalized SWNT sensors is highly sequence dependent. Different sequences of DNA can selectively respond to certain chemicals. Samuel and their group studied four kinds of homo-DNA functionalized SWNT field effect transistors (SWNT-FETs) for detecting several gaseous analytes [260]. The response to trimethyl amine (TMA) followed the trend d(G)_{21}-SWNT > d(A)_{21}-SWNT > d(C)_{21}-SWNT > d(T)_{21}-SWNT. They also suggested a comparable trend of d(G)_{21} > d(A)_{21} > d(C)_{21} > d(T)_{21} exists for DNA-SWNT binding affinities. They concluded that stronger binding between DNA and SWNT will be formed with a greater amount of DNA adsorbed to SWNT surface. This provides a more hydrophilic...
environment around the hydrophobic SWNT core and thus facilitates the adsorption of polar analytes. The work of A. T. Charlie Johnson and his colleagues also confirmed this conclusion as the nanosensors functionalized with strands comprised of purines (A or G) were responsive to methanol vapor and TMA while the ones with strands of pyrimidines (C or T) were not [274]. However, it was also displayed that for SWNT sensors with multi-oligomer DNA sequences, the cases are complex and cannot be easily explained. Besides, the sequence length of DNA decorated on the SWNT sensors also affects the sensing performance. Yu et al. found that the decoration of 24 bases of poly-G ssDNA on SWNTs led to the highest resistance change when the sensors were exposed to methanol and IPA vapors [261]. But in this paper, we found the optimum length of DNA GT decorated on SWNT sensors for detection of acetone and HCl is 32 and 8, separately. So the sensing performance must also be analyte related other than sequence dependent.

All DNA decorated SWNT sensors demonstrated better sensing performance than bare SWNT nanosensors towards both methanol and acetone. Acetone is a polar organic compound. A possible reason for the DNA based sensitivity enhancement of SWNT nanosensors to polar molecules could be that the decoration of DNA has converted the SWNT surface properties from hydrophobic to hydrophilic. Zhao et al. suggested that the hydrophobic groups on the nitrogenous bases of oligonucleotides tend to bind onto the surface of SWNTs, while the hydrophilic phosphate groups along the backbone of oligonucleotides do not bind to SWNTs [275]. Thus after DNA was coated onto the carbon nanotubes, the outer surface of the DNA-SWNT structure would be covered by the hydrophilic backbones of DNA. This hydrophilic surface environment would then attract polar molecules including methanol and acetone. On the other hand, although HCl is also a polar molecule, the way SWNT nanosensors respond to HCl is different. When SWNT nanosensors were exposed to HCl, there existed the interaction of HCl with adventitious impurities and the intercalation of the SWNT bundles with HCl molecules. The interaction of HCl with adventitious impurities may change the intertube contact resistance. In addition, some of the binding sites were
irreversible. Once they were bonded to nanotubes, they were very hard to get desorbed. Moreover, the variety of DNA sequences may also change the 3D structures of DNA-SWNT. This explains the variation of the sensing results between methanol/acetone and HCl.

Our objective is to find which DNA sequence and length is able to identify a specific chemical, therefore a fixed concentration of 50 ppm was applied in all tests here. The DNA sequences used in this paper were chosen based on prior work in our group. If we want to try many possible DNA sequences to find the specific one for our analyte, it would be very costly and time consuming. Thus, a well-organized and convenient method to screen out the proper DNA sequence for specific use is required. We are exploring computational system, e.g., information theory (IT) and molecular dynamics (MD) for simulating and analyzing these sensing processes to clarify the mechanisms behind this sequence-dependent sensing performance.
CHAPTER 7

DNA SCREENING FOR TARGETED MOLECULAR BIOMARKERS USING MOLECULAR DYNAMICS SIMULATION

DNA biochemical sensors can be utilized to detect certain disease biomarkers present in individuals’ breath, for instance, acetone and ethanol for diabetes and H$_2$S for cardiovascular diseases. They can also be applied in the detection of toxic or explosive gases like HCl in the air for air quality monitoring. Before experimenting on thousands or millions potential DNA segments, we have conducted full atomistic steered molecular dynamics (SMD) simulation of the interactions between different DNA sequences with targeted molecules to screen out the best sensing-performance nucleobases. Through the Jarzynski Equality, we were able to rank the approximate free-energy of interaction between four single DNA nucleotides (Adenine (A), Guanine (G), Cytosine (C), and Thymine (T)) on both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) with acetone, ethanol, H$_2$S and HCl. By sampling forward and reverse interaction paths, we were able to compute the free-energy profiles of the eight aforementioned systems for the four
targeted molecules. We found that dsDNA react differently than ssDNA to the targeted molecules, requiring more energy to move the molecule close to DNA as indicated by the potential of mean force (PMF). Comparing the PMF values of the different systems, we obtained the best DNA bases and relative ranking for the detection of each molecule. In the same way, we could generate a library of DNA sequences for the detection of a wide range of chemicals. A DNA sensor array built with selected sequences differentiating many disease biomarkers can be used in disease diagnosis and monitoring.

7.1 INTRODUCTION

Through the relative efficiency of exchanging nucleobases to vary sequence [276-279], DNA provides an ideal platform for diverse molecular engineering. Indeed, it is through this sequence variation that all biological cells and tissues arise. From a technological perspective, the interactions between DNA and small molecules have been exploited to build biochemical sensors for disease diagnosis [93, 280-283] and detection of explosives [38, 262], and they have demonstrated very high chemical sensitivity, molecular selectivity and good stability. However, the particular sequences used in these studies were either from earlier experience or literature reports. The question arises: given an arbitrary target molecule, can an ideal DNA sequence be designed to maximize interaction? Here, we tackle the first step of this question, probing the interaction of small molecules with target nucleobases – *e.g.*, the basic building blocks of single-stranded and double-stranded DNA – *via* a computational screening approach. This is the first necessary step in sequence optimization, to be used as a protocol for DNA sequence design *in silico* for specific applications. To the best of our knowledge, no other group has reported how to design DNA sequences to achieve the best detection results for particular molecules.

The molecules of interest we choose for the current study are common *biomarkers* – measurable indicators of pathological conditions generated by the body. For example, for diabetes the most common biomarker is glucose levels in the blood (which effectively defines the metabolic
pathology). However, both acetone [227, 284-285] and ethanol [118, 285] are biomarkers for diabetes found in breath. Acetone, for example, is reported to be less than a few hundred ppb (by volume) in the breath of healthy individuals [286] while for diabetic patients, acetone concentration can reach 560 ppm or even >1000 ppm [287]. In addition, hydrogen sulfide (H₂S) is a probable indicator of bad breath, and more importantly, a potential biomarker for a variety of cardiovascular diseases [288] and chronic pancreatitis [289]. Finally, hydrogen chloride (HCl) is such a toxic gas in the air that exposure to 1.8 ppm of it can cause subject’s upper respiratory system symptoms of sore throat and nasal discharge, and 20 ppm is recommended as a level beyond which can cause severe adverse effects [290]. DNA sensors which are capable of detecting these chemicals with high sensitivity and selectivity substantiating a great potential in disease diagnostics and monitoring, as well as in air quality monitoring.

Paring nucleobases to molecules will enable the possibility to map an array of DNA sequences for reliable detection of several particular biomarkers of one specific disease, and provides a new paradigm of design, development, and application of advanced engineering material systems, combining computational approaches, optimization methods, and DNA informatics. To this end, full atomistic molecular dynamics (MD) can play a major role in this process, acting as a high resolution “virtual microscope” to characterize DNA/biomarker interactions with high fidelity, complementing experimental results [291-292]. This is in line with the current Materials Genome Initiative [293], using computational means as a method to screen materials interactions, with a unique biomolecular focus, creating an expanding library or databank of DNA/molecular interactions.

Here, as a first screening protocol to select the promising DNA sequences, we use full atomistic molecular dynamics (MD) simulation, which has been widely applied in biomolecular systems [294] to probe the molecular interactions between short DNA models systems and small molecules. In silico, the DNA sequences can be selected, refined, and optimized prior to synthetic efforts, in an efficient manner. Moreover, simulation can efficiently extract interaction parameters
(e.g., the energy of interaction, H-bonding, etc.) difficult to measure experimentally [295-296]. It is believed that computational methods can be exploited to better interpret the selection, use, development, and discovery of materials, with a goal to achieve rapid and robust acquisition, management, analysis, and dissemination of diverse materials’ data [291, 296-297]. In theory, rather than screening thousands/millions of potential DNA sequence candidates, MD simulation can be applied to select optimized DNA sequences (highest sequence-chemical sensitivity/affinity) to detect one particular chemical. Simulations will not only help bridge the gap between computer simulation and the experiment but also provide unprecedented insight into the behavior of the atomistic interactions between DNA and molecules.

Specifically, we implement MD simulations to screen binding/unbinding affinity of the four introduced biomarkers (acetone, ethanol, hydrogen sulfide, and hydrogen chloride) to the four possible nucleobases (A, C, G, and T) of both single-stranded and double-stranded DNA (ssDNA, dsDNA). We note the intent is neither to directly quantify the energetics of interaction nor to quantify the specific interaction pathways (which could be the focus of future work). Rather, we wish to demonstrate a screening/ranking procedure, to select the “best” nucleobase for biomarker interaction. From this base, an optimized sequence can then be initiated, leading to sequence-specific DNA sensors. In the following sections, we will introduce the MD simulation method in detail, the yield quantitative information about the binding potentials of the DNA-molecule systems, and important insights we have obtained into the biological processes.

7.2 THEORY AND METHOD

Molecular dynamics (MD) is a computational method describing equilibrium and dynamics properties of an atomistic system. It generates the configurations of a system by integration of Newton’s laws of motion through calculating the time dependence of the molecular system, and also provides information at the microscopic level - e.g., atomic positions, velocities and energetics. It helps to understand the properties of assemblies of molecules in terms of their structures and the
microscopic interactions between them. MD serves as a complement to conventional experiments, enabling us to learn something new, something that cannot be found out in other ways. For example, it is near impossible to bind a small molecule with a specific nucleobase in a random DNA sequence experimentally, but relatively easy by simulation. We proceed to describe the molecular models, the MD force field, and the interaction assessment procedure via steered molecular dynamics (SMD).

### 7.2.1 MOLECULAR MODEL

The first step in MD simulation is an accurate construction of the atomistic geometry including a clear definition of the atomistic location, the element type (i.e., the atomic mass and associated chemical properties), and the bond connectivity among all atoms. In our study, it includes single- and double-stranded DNA structural arrangements consisting the definition of each individual nucleotide, targeted biomarker, and surrounding water molecules. DNA (deoxyribonucleic acid) is a long linear polymer built up from many monomer units called nucleotides, consisting of three components: a sugar, a phosphate, and one of the four bases-either adenine (A), guanine (G), cytosine (C), or thymine (T). The backbone of DNA strands is made from alternating phosphate and deoxyribose, so we would use symbols A, G, C, and T to represent the four types of nucleotides. Two of the bases are derivatives of purines-A and G and two of pyrimidines-C and T (Fig. 7.1a). By definition, ssDNA is a single strand lacking base pairs, while dsDNA has two chains wound in a helical structure with the base pairs-G with C and T with A on the sugar-phosphate backbones (Fig. 7.1b). The double helix is more stable due to the hydrogen bonds between nucleotides and base-stacking interactions among aromatic bases [298]. Both constructed DNA systems consist of 24 base nucleobases, constructed in a random manner with the ssDNA sequence: GTCTTACGCTAGCTGGGATTACG. The dsDNA has one strand with the same sequence and the other of its complementary sequence. The sequence is consistent for all simulations.
Fig. 7.1. Chemical Schematics: a) structures of DNA bases-A, G, C and T; b) DNA base pairs-G=C and A=T; and c) structures of chemicals-acetone, ethanol, H₂S and HCl.

The molecules we selected (Fig. 7.1c) are not only biomarkers for diseases or indicator of air quality but also belong to different functional groups which can greatly help us sum up generate rules in the interactions between DNA nucleotides and small molecules. Acetone is the simplest ketone which has two methyl groups at the two ends of a carbonyl group (C=O) forming a nucleophilic property at the oxygen end and an electrophilic property at the sp² hybridized carbon end. It is a hydrogen-bond donor. Ethanol is a 2-carbon alcohol with a nonpolar end (CH₃) and a very polar end (OH) which enables hydrogen bonding. Hydrogen sulfide is a polar molecule but with very low solubility. Hydrogen chloride is very soluble partly because of its high polarity. These molecules were constructed manually based on known chemical geometry.

Once the molecules were assembled in a single system (Fig. 7.2a), the ssDNA or dsDNA with biomarker were solvated in waterbox of approximate dimension 40Å × 90Å × 90Å using the explicit TIP3P explicit water model - a three-site rigid molecule [299] - as implemented in CHARMM [300]. The large size of the water box is constructed to make sure the whole system is solvated/saturated during the simulation period (Fig. 7.2b) with adequate screening. Note that no counter-ions are included in the current simulation, as the selected biomarkers are not
predominantly electrostatic in nature, and the effect of electrostatic screening can be assumed negligible across the molecules of choice (which is not universally true).

Fig. 7.2. Visualization of the simulated system: a) Full atomistic model of 24 nucleotide-DNA strand-small molecule (Acetone in particular); b) Complete simulation system, with 40Å × 90Å × 90Å periodic water solvation box consisting of 10, 815 water molecules.

7.2.2 ATOMISTIC FORCE FIELDS (CHARMM/CVFF)

There are multiple possible force fields (potentials) available to evaluate the inter-atomic interactions which describe the chemical properties and they play an important role in the accuracy of the computational modeling studies via proper description of the atomic interactions. In spite of many force fields available in the literature for different types of interactions among atoms, in terms of DNA, we select the well-proven CHARMM force field - a nonreactive potential with a basis on
harmonic potentials [301-303]. CHARMM has been parameterized to reflect the structure of DNA [304-305]. For the biomarkers, we use parameters from the consistent valence force field (CVFF), which has also been applied in the simulation of polymers, nucleic acids, and organic molecules [306]. The formulation of both CHARMM and CVFF are similar - enabling seamless integration - where the total energy of the system is represented as a sum of covalent (bond, angle, dihedral, and improper) and noncovalent (van der Waals, Coulombic) contributions:

\[
E = \sum_{\text{bonds}} E_b + \sum_{\text{angles}} E_\theta + \sum_{\text{dihedrals}} E_\phi + \sum_{\text{impropers}} E_\omega + \sum_{\text{pairs}} E_{\text{vdW}}
\]

(7. 1.)

The pair potential parameters of the van der Waals interactions (Lennard-Jones pair potential) between different atom types are mixed according to the geometric mean and arithmetic mean for the energy and distance respectively:

\[
\varepsilon^{AB} = \sqrt{\varepsilon^A \varepsilon^B}
\]

(7. 2a)

\[
\sigma^{AB} = \frac{1}{2}(\sigma^A + \sigma^B)
\]

(7. 2b)

Hydrogen bonds are implicitly included in the Lennard-Jones 12:6 formulation. Both CHARMM and CVFF parameterizations utilize harmonic potentials for covalent interactions such as bond, angle, dihedral, and improper terms with the hypothesis that inter-molecule interactions are significantly weaker than that of covalent bonds.

For all simulations, Large-scale Atomic/Molecular Massively Parallel Simulator (LAMMPS; [http://lammps.sandia.gov/]), an open-source molecular dynamics software package is used to perform all MD simulations [307-308]. After manually constructing the DNA-chemical system geometry, a minimization of energy for the entire system is done prior to dynamic simulation using a conjugate gradient algorithm to ensure a lowest total potential and stable initial structure. After minimization, unconstrained molecular dynamics simulation over 100ns at 300K using a NVT ensemble is performed to equilibrate the system prior to initiation of SMD. The constraints of the
simulation are then be defined and applied to the system to control the simulation process where the computational experiment can be accurately performed.

### 7.2.3. STEERED MOLECULAR DYNAMICS (SMD)

To induce interactions between DNA with targeted molecules we implement a non-equilibrium steered molecular dynamics (SMD) approach, which approximately mimics an AFM nanomechanical loading experiment by applying a directional spring force to an objective molecule. SMD is a novel approach to study the dynamics of binding or unbinding events in biomolecular systems [309], revealing the details of molecular interactions in the course of unbinding [310-311] and providing important insights of the binding mechanisms underlying these processes. The primary advantage of non-equilibrium SMD over conventional equilibrium MD methods is the possibility of inducing relatively large conformational changes in molecules within the nanoscale time scales accessible to computation.

Computationally, the SMD method applies a moving spring force (Fig. 7.3) so that the molecule can behave in a manner not obtained by either force or displacement loading alone, allowing induced conformational changes in a system along a prescribed reaction vector. The driving force applied to the atom group is:

\[
F_{SMD} = k_{spring}(R - R_0)
\]

(7.3)

where \( k_{spring} \) is the spring constant, and \( R_0 \) is the distance from the end of spring to an arbitrary tether or target point. A constant velocity, \( v \), is assigned which monotonously increments or decrements the distance \( R \) towards the tether point. The DNA molecule, either single-stranded or double-stranded, is set at one end of the solvation box, and the SMD force is applied at the geographical center atom of the biomarker. As the DNA molecule is relatively large, its movement during the simulation can be neglected. The small molecules are pulled towards the middle of one particular nucleotide each simulation, providing the direction of spring velocity. Total force and the PMF
values during the SMD simulations can then be plotted against the distance between the biomarker and DNA.

![Schematic of SMD simulation](image)

Fig. 7.3. Schematic of SMD simulation. General constant velocity SMD approach where macromolecule is connected with harmonic spring with defined stiffness, $k_{spring}$, and a fixed velocity, $v_{constant}$, towards a target coordinate $(x, y, z)$; in this case, the target is a single nucleobase (A, C, G, T) of a 24-based ssDNA/dsDNA.

We first decide an appropriate spring constant at which varying the velocity of the spring doesn't change the applied force and yield PMF values. We can then maintain the constant spring constant and assign a modest pulling velocity throughout the investigation for computational efficiency. It is known that the spring constant can affect the total energy landscape in physical systems [312-313], however, this effect is not studied further here. To define an appropriate spring constant in the SMD simulation, $k_{spring}$, we used G-nucleotide on ssDNA-ethanol system to test different $k$-values at various pulling velocities. After testing different $k$-values ranging from 0.1 to 10, we found $k = 10 \text{ kcal/mol/Å}^2$ is a good choice (Fig. 7.4). For the total force, a clear drop was observed from approximately 23Å to 10Å, where the interaction between the two molecules started. The more the applied force drops, the stronger attraction force between the two molecules indicating a higher affinity between them. Once the distance between them became very small, less than 5Å, the two molecules started repelling each other leading to an exponential increase of both
the total force and the energy. The total force (Fig. 7.4a) and accumulated PMF (Fig. 7.4b) provided almost the same profiles at different pulling velocities ranging from 0.00005 to 0.001 Å/fs. Thus, we use \( k_{\text{spring}} = 10 \text{ kcal/mol/Å}^2 \) (6.95 N/m) and \( v_{\text{constant}} = 0.0001 \text{ Å/fs} \) (10 m/s) as the setup for SMD simulation.

**Fig. 7.4.** SMD simulation results of G-nucleotide in ssDNA-ethanol system at \( k_{\text{spring}} = 10 \text{ kcal/mol/Å}^2 \) with various pulling speed from 0.00005 to 0.001 Å/fs: a) the total force in the direction of pull; b) the accumulated PMF.

### 7.2.4 JARZYNSKI EQUALITY AND BIOMARKER INTERACTION RANKING

Here, we wish to probe the interaction between a biomarker. Despite plentiful modeling methods for such interactions [314], little is known \textit{a priori} about processes of binding and unbinding, limiting any predictive (or design) power. Presently, the prevailing point of view concerning computer simulations describing binding and determining binding affinities is to strive for the ideal of reversibility, as in umbrella sampling and free energy perturbation [315-321], with the hope that artifacts induced by the finite rate of conformational changes can be neglected. Reaching this ideal, however, requires extremely slow manipulation and, therefore, prohibitively expensive simulations. An SMD simulation is a non-equilibrium process, which accepts irreversibility, ceding for the present time accurate evaluation of binding affinities and potentials of mean force, but gaining access to biologically relevant information related to non-covalent bonding.
The concern that thermodynamic potentials cannot, even in principle, be obtained from irreversible processes has been proven unfounded by the Jarzynski Equality [322]. The Jarzynski Equality (JE) is a statistical mechanical equation that relates the change in free energy, $\Delta F$, between two equilibrium states via a non-equilibrium process. Here, this is a bound biomarker (state A) with an unbound biomarker (state B) via SMD. In a quasi-static process, the work, $W$, done on a system from $A \rightarrow B$ can be said to be:

$$\Delta F = F_B - F_A \leq W \quad (7.4)$$

when the system transitions from A to B infinitely slowly. The JE, on the other hand, remains valid regardless of the process speed, where:

$$\exp\left(-\frac{\Delta F}{k_B T}\right) = \langle \exp\left(-\frac{W}{k_B T}\right) \rangle \quad (7.5)$$

where $k_B$ is Boltzmann’s constant and $T$ the temperature. The angled brackets, $\langle \rangle$, indicates an average over all possible realizations of the external process that takes $A \rightarrow B$. This identity connects the ensemble average of an exponential of the total work $W$ performed on the system during a non-equilibrium transition from one state to another to the free energy difference $\Delta F$, which is an equilibrium property, between the two states. In general, $W$, depends on the specific initial micro- (or nano-) state of the system, where the ensemble of multiple states implies $\Delta F \leq \langle W \rangle$. Since its derivation, the JE has been verified to be an accurate (although non-exact [323]) approach in both experiments [324] and simulations [325] of biomolecules and small molecules.

To properly capture the free energy describing the conformational space of the binding event and ensure the equality in eq. 7.5, the proposed SMD simulations would need to include a very large statistical sample of both multiple initial conditions and multiple directions of the binding vector. This would enable an accurate calculation/prediction of $\Delta F$. For ranking purposes, however, this degree of accuracy is unnecessary. The JE implies an average of the work over all phase space
trajectories from one state to another. Here, we probe one approach/trajectory per biomarker/nucleobase pairing. However, the trajectory is *equivalent* for all systems. Thus, we assert that if we apply an equivalent microstate between systems, while $\Delta F$ cannot be accurately determined, $W$ can be sufficiently approximated to rank the attained approximate energies, $\Delta \hat{F}$, of the interactions. In simple terms, asserting the same initial conditions will result in similar deviations in free energy, such that the ranking of $\Delta F$ and $\Delta \hat{F}$ for each biomarker/nucleobase pair does not change.

### 7.3 RESULTS AND DISCUSSION

We conducted SMD simulations of the interaction between DNA and targeted molecule to investigate the affinity of the molecules on different DNA nucleobases. Specifically, we study the interactions between single nucleotide (A or G or C or T) on both ssDNA and dsDNA with the targeted molecules (acetone, ethanol, H$_2$S and HCl), or 32 systems in total. The affinity between the DNA nucleotides and chemicals can not only be assessed via energy profiles but also be simply visualized by the geometry. Snapshots of the ssDNA-HCl model (targeting G-nucleotide) are shown in Fig. 7.5. As a representative example, interactions between four different nucleotides on both ssDNA and dsDNA with ethanol molecule are displayed in Fig. 7.6a-b and Fig. 7.6c-d respectively.
Fig. 7.5. Representative SMD simulation snapshots of G-nucleotide in ssDNA-HCl system at pulling speed of 10 m/s at a) 60 picosecond, b) 138.3 picosecond, c) 180 picosecond, and d) 203.25 picosecond. It is noted that the water molecules with their hydrogen bonds are not shown (but exist all the time) to highlight the interaction between DNA nucleotide and HCl molecule.

7.3.1 BIOMARKER INTERACTION WITH SSDNA

For different nucleotides applied in ssDNA-ethanol systems, clear force drops were all observed when ethanol molecule was pulled towards T, C and G nucleotides, while the pulling force of ethanol towards A nucleotide almost remained the same until the two molecules started repelling each other (Fig. 7.6a). It seems ethanol is not attracted to A nucleotide as much as to the other nucleotides according to the force profiles, however, by looking into the accumulated PMF values which reveals the energy profiles, the energy of A, T and G nucleotides and ethanol systems was much lower than that of C nucleotide-ethanol system. Therefore we conclude the affinity of ethanol with different nucleotides on ssDNA follows a trend of A=T=G>C. Ethanol is a polar molecule with a hydroxyl functional group at the end. It can interact with carbonyl and amine groups forming hydrogen bonds. Adenine has three tertiary amines, Guanine has two tertiary amines and one carboxyl group, Thymine has two carboxyl groups, and Cytosine only has one
carboxyl group (Fig. 7.1). It is also known aromatic ring can weaken the electron-donor property of O (oxygen) and N (nitrogen), and primary and secondary amines can form hydrogen bonds with hydroxyl group as well. Therefore it makes great sense that the attraction between ethanol and single-stranded DNA follows a trend of A=T=G>C. This simple ranking procedure represents a great feasibility to select the optimal DNA nucleotide/nucleotides towards particular biomarkers without the need of sophisticated computational chemistry (e.g., molecular system energy) or conducting a lot of conventional experiments.

Fig. 7.6. SMD simulation results of A, T, C, and G nucleotides in ssDNA-ethanol system (a and b) and in dsDNA-ethanol system (c and d) at $k_{spring} = 6.95$ N/m with pulling speed at 10 m/s: a) and c) the total force in the direction of pull; b) and d) the accumulated PMF.

7.3.2 BIOMARKER INTERACTION WITH DSDNA
For different nucleotides used in dsDNA-ethanol systems, more fluctuations could be seen in the pulling force profiles, and this is due to the complexity of the dsDNA structure which has complementary pairing interactions within the two strands of DNA molecules. Guanine and Cytosine can pair together forming three hydrogen bonds while Adenine and Thymine can form two hydrogen bonds. Since the DNA double helix is stabilized by the hydrogen bonds between the nucleotides and by the base-stacking interactions among aromatic nucleobases, the interactions between nucleotides and ethanol molecules were weakened. This is depicted as the increase of PMF levels compared to that of ssDNA-ethanol system. It is concluded from the energy profiles that the affinity of ethanol with different nucleotides on dsDNA follows a trend of A>G>C>T. Single-stranded DNA is more favored here because of its higher specificity. For instance, one type of nucleotides on one strand of a dsDNA shows the weakest interaction with one particular molecular biomarker while its pairing nucleotide may have the strongest interaction with this biomarker. Thus, the sensitivity and specificity of dsDNA chemical sensors are both reduced compared to ssDNA ones.

Similarly, for DNA-H2S systems, we obtained SMD simulation results as shown in Fig. 7.7. H2S is a three-atom polar molecule (Fig. 7.1c), it is much smaller and lighter compared to acetone and ethanol. Thus the interaction between DNA and H2S is more active which is consistent with the more fluctuations of the pulling force observed in both ssDNA-H2S and dsDNA-H2S systems. It also happened to eight DNA-HCl systems.
Fig. 7.7. SMD simulation results of A, T, C, and G nucleotides in ssDNA-H$_2$S system (a and b) and in dsDNA-H$_2$S system (c and d) at $k_{spring} = 6.95$ N/m with pulling speed at 10 m/s: a) and c) the total force in the direction of pull; b) and d) the accumulated PMF.

7.3.3. RANKING OF BIOMARKER INTERACTION WITH DNA

Ranking of the four molecular biomarkers- acetone, ethanol, H$_2$S and HCl, interaction with four different DNA nucleotides- A, T, C, and G on both ssDNA and dsDNA is depicted in Fig. 7.8. Summary of the affinity of four chemical molecules with different DNA nucleotides is provided in Table 7.1. The criteria applied to generate the rankings include first - the PMF value before the two molecules start repelling each other; second - the closest distance the molecular biomarker can approach DNA nucleotide; and third - the total force drop when two molecules are pulled together.

Here we focus more on the interaction between nucleotides on ssDNA with molecular biomarkers...
due to the stronger interaction and higher specificity as compared to those observed with dsDNA. Adenine nucleotide shows the highest affinity with acetone molecules, and this can be attributed to the carbonyl group (C=O) in the acetone molecule which reacts with the amine group (-NH2) in adenine. In spite of the existence of similar amine groups in guanine, cytosine, and thymine nucleobases, the effect of electron-donating is weakened by the carboxyl groups in cytosine and thymine nucleobases, especially by the aromatic ring presented in the guanine nucleobase. Thus, the affinity between acetone and different nucleotides follows the trend A > C ≥ T > G when considering single-stranded DNA.

The interaction between small molecules and double-stranded DNA is much more complicated due to the hydrogen bonds within the dsDNA and its complementary pairing properties. The ethanol molecule has a very polar end- hydroxyl group, for example, which interacts with both carbonyl groups and amine groups forming hydrogen bonds. With the weakening effect from the aromatic ring and the enhancing effect from primary and secondary amine groups, it makes great sense that the affinity between ethanol and single-stranded DNA follows a trend of T ≥ G > A > C. H2S is a polar molecule, and S (sulfur) is an electron-rich element and a homologue of O (oxygen). Based on the principle of the dissolution in the similar material structure, adenine nucleotide should have the least affinity with ethanol molecule, and it is also the result from our simulation which confirms the practicality of our computational approach. HCl is a very soluble due to its high polarity. It is hard to predict the interaction between HCl and nucleotides, but MD simulation has provided with a clear trend of the affinity between these two molecules.

Table 7.1. Affinity strength ranks of DNA nucleotide-chemical systems

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Ranked Affinity with ssDNA</th>
<th>Ranked Affinity with dsDNA</th>
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<tbody>
<tr>
<td>Acetone</td>
<td>A&gt;C&gt;T&gt;G</td>
<td>A&gt;G&gt;T&gt;C</td>
</tr>
<tr>
<td>Ethanol</td>
<td>T≥G&gt;A&gt;C</td>
<td>A&gt;G&gt;C&gt;T</td>
</tr>
<tr>
<td>H2S</td>
<td>G&gt;T&gt;C&gt;A</td>
<td>C&gt;T&gt;G&gt;A</td>
</tr>
<tr>
<td>HCl</td>
<td>C&gt;G&gt;A&gt;T</td>
<td>A=G&gt;C&gt;T</td>
</tr>
</tbody>
</table>
Due to a lack of isolated DNA with biomarker systems, we resort to a comparison between the simulated results and a known DNA + carbon nanotube sensor. In our earlier experiments on sensing trace amount of chemicals in vapor [326], we designed and fabricated a wireless sensor array based on ssDNA-decorated single-walled carbon nanotube (SWNT) on micro devices. Microelectrodes with 3 µm gap were fabricated by photolithography followed by sputtering Cr/Au (20 nm/150 nm) layer onto a silicon oxide substrate. Then ultrathin films of SWNTs were assembled between pairs of microelectrodes by a low temperature and also low cost DEP assembly process. ssDNA of different sequences were noncovalently bonded to the SWNT surfaces. Changes in resistance indicate the interaction between DNA and gas molecules. The sequences used include

DNA 24A: AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
DNA 24Aa: amine-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-amine
DNA 24GT: GTGTGTGTGTGTGTGTGTGTGTGT
DNA 24Ma: amine-GTCTTACGCTAGCTGGGCATTACG-amine

Fig. 7.8. Ranking of molecular biomarker- acetone, ethanol, H₂S and HCl, interaction with A, T, C, G nucleotides on a) ssDNA; and b) dsDNA indicated by PMF values.
By introducing the carbon nanotube, the presence of the hydrophobic nanotube sidewall directly adjacent to the nucleotides (which are adsorbed on the sidewall via their hydrophobic bases) may significantly change the binding environment for a given biomarker – however, we can qualitatively compare the results. Taking acetone sensing as an example, the response ranking of the ssDNA-SWNT sensors towards acetone follows 24Ma > 24A > 24GT > 24Aa, which slightly differs from the simulated ssDNA-acetone interaction result (A > C ≥ T > G, Table 7.1). There are two factors which determine the chemical sensing performance using our sensor array. There are binding between SWNT and ssDNA and interaction between ssDNA and chemicals. Khamis et al. has reported that the affinity of homo-ssDNA wrapping around SWNT follows a trend of G > A > T > C [260], and this means ssDNA sequences with more G nucleotides would bind more onto SWNT sensor. Stronger binding between ssDNA and SWNT can create a more hydrophilic environment around the hydrophobic SWNT core and facilitate the adsorption of acetone molecules. On the other hand, the interaction between ssDNA and acetone follows A > C ≥ T > G which means A nucleotide reacts stronger with acetone. DNA 24Ma functionalized SWNT sensor has a sequence of mixed A, G, C, and T nucleotides, and it has demonstrated a stronger response compared to DNA 24A-SWNT sensor. It implies that adding G nucleotides into DNA sequence can improve the acetone sensing performance by increased binding between DNA and SWNT. DNA 24GT decorated SWNT sensor displayed the second least affinity towards acetone, and it is consistent with the simulated ssDNA-acetone interaction ranking. The fact that DNA 24 Aa decorated SWNT sensor revealed the least response to acetone is likely due to the intermolecular interactions between DNA molecules at the amine group ends. Though we don’t have experimental results on all four homo-ssDNA decorated SWNT sensors for acetone sensing, the limited ranking of responses of ssDNA-SWNT sensors on acetone is very comparable to the SMD simulation results combined with the affinity ranking of SWNT and ssDNA. Thus, we would high confidently recommend a DNA sequence consisting most of G and A nucleotides-decorated SWNT sensor for highly
sensitive acetone detection based on DNA G nucleotide’s best binding ability to SWNT and DNA A nucleotide’s best interaction with acetone molecule and good affinity to SWNT as well.

7.3.4. UNBINDING SIMULATIONS

A condition of the JE is that it holds for the reversible process – e.g., the work necessary for transition from $A \rightarrow B$ should be equivalent to the magnitude of the work necessary for $B \rightarrow A$, or $|W_{AB}| = |W_{BA}|$. That being said, as before, the equivalence presumes a full statistical sampling of all possible microstates for each transition. Moreover, the initial microstates differ for each transition. Regardless, between biomolecular systems, we again assert that deviations from exact energetic values would be consistent, as each biomarker is subjected to the same microstate, as such the ranking of unbinding simulations should reflect the physical molecular affinities.

As such, we also simulated the pulling-forward (binding) and following pulling-away (unbinding) processes to confirm the affinity of the different biomarkers with different DNA nucleotides. Taking ssDNA-acetone system for example (Fig. 7.9), the absolute value of PMF increased when acetone was pushed away indicating there was an attraction force between acetone and DNA (Fig. 7.9b). A higher absolute value of PMF when pulling acetone molecule away from DNA means a higher affinity between these two molecules. As shown in the lower part of Fig. 7.9b, the pulled away $|\text{PMF}|$ follows the trend $A>C>T=G$ which confirms the affinity strength rank obtained through the pulling forward approach (Table 7.1).
7.3.5. EFFECT OF NUCLEOBASE NEIGHBOR

Finally, while we target single nucleobases, the ultimate goal is to optimize the entire DNA sequence for biomarker interaction. To indicate preliminary effects of sequence variation, we probe the interaction between nucleobases with variant neighbors. As indicated in Fig. 7.10, for the same nucleotide-G, the PMF values vary on the neighboring nucleotide types. The ssDNA-acetone system with G nucleotide positioned between G-G nucleotides provides the lowest energy at the closest distance while the energy increases when G is positioned between A-C, G-C, or T-G nucleotides. This means the interaction between neighboring nucleotides and the studied nucleotide also affects the affinity of the studied nucleotide-small molecule system. Clearly, the effect of neighbors will be dependent on the size of the selected biomarker, as well as the screening environment (e.g., presence of ions) which is not varied here, and suggests additional future investigation. Further study of the effect of DNA sequence and length will be carried out. Validation of the simulation results through conventional experiments is also in progress.
Fig. 7.10. SMD simulation results—the accumulated PMF of G nucleotides with different neighboring nucleotides on ssDNA-acetone systems at $k_{\text{spring}}=6.95$ N/m, $v_{\text{constant}}=10$ m/s.

### 7.4 CONCLUSION

#### 7.4.1. DNA SEQUENCE DESIGN FOR SPECIFIC APPLICATIONS

In order to select the optimal DNA sequences in building DNA biochemical sensors for breath analysis or air quality monitoring, we conducted SMD simulations of the interactions between different DNA sequences and our targeted molecules. Acetone and ethanol are breath biomarkers for diabetes, while elevated H$_2$S level can indicate cardiovascular diseases or chronic pancreatitis. HCl is highly toxic and can cause immediate danger to life with only 50 ppm concentration. We studied the interaction between four single DNA nucleotides (A, G, C, and T) on both ssDNA and dsDNA with acetone, ethanol, H$_2$S and HCl. In SMD simulation, the center-of-mass of small molecules was pulled at a certain velocity towards one particular DNA nucleotide. The mechanical work of pulling it forwards (forward pulling path) and backwards (reverse pulling path) at a number of points was measured during this process. By sampling these forward and reverse paths, we were able to know the equilibrium distance and to accurately compute the free-energy profiles of the eight aforesaid systems for each targeted molecule. Four DNA nucleotides
on dsDNA were found to react differently to the targeted molecules than on ssDNA, requiring significant higher energy to move the molecule close to DNA than the later. Comparing the PMF values of the different systems, we obtained the optimal DNA bases/sequences for the detection of each molecule: Adenine for acetone, Guanine or Thymine or Adenine for ethanol, Guanine for H₂S, and Cytosine for HCl. Taking the affinity ranking of SWNT and ssDNA into account, the simulation results are in good agreement with our earlier sensing results using ssDNA-SWNT sensors. A library of DNA sequences for the detection of a wide range of chemicals can be easily generated via this method. A DNA sensor array built with selected sequences differentiating many disease biomarkers or indicating harmful gases in the air can be used in disease diagnosis and monitoring and air quality monitoring as well.

7.4.2. DESIGNER BIOMATERIALS FOR NEXT GENERATION BIOMARKERS

DNA nanotechnology has already become an interdisciplinary research field, with researchers from chemistry, materials science, computer science, biology, physics and medicine coming together to tackle important problems. As the field is progressing rapidly, we believe that exciting new directions will emerge well beyond the limited set described here. Such bottom-up computational approaches, capable of understanding atomistic-scale interactions in biomaterials, provide an outstanding platform to screen/design high-performance biomaterials for cheap and efficient medical diagnosis, drug design, or point-of-care assessment.

Biomaterials have a huge impact on health care [327-330], and are being widely used in medical devices [331-333] and drug delivery systems [334-336]. Advancements have been made in the understanding of the natural functions of biological materials and systems, and more importantly, in designing and synthesizing new functional biomaterials useful in clinical applications [337-340].
The necessary design space of materials and molecular compounds is clearly vast, with only a small percentage of possible materials being exploited in useful technologies. For complex interactions, high-throughput screening of molecular compounds has been the traditional *brute force* approach, and commonly applied in drug discovery [341-342]. Recently, drug discovery has moved toward more rational strategies based on our increasing understanding of the fundamental principles of protein-drug or cellular-drug interactions. With advancements of simulation and modeling, virtual approaches to screening have been successfully implemented with a goal of reducing large compound databases and to select a limited number of promising candidates for drug design [343-345]. Indeed, high-throughput computational materials design is an emerging area of materials science. By combining advanced thermodynamic and electronic-structure methods with intelligent data mining and database construction, and exploiting the power of current supercomputer architectures, materials scientists can generate, manage and analyze enormous data repositories for the discovery of novel materials [346-347].

It is now believed that computational methods can be exploited to better understand the use, selection, development, and discovery of materials, with a goal to achieve high-speed and robust acquisition, and dissemination of diverse materials data. Used as a screening tool, biomaterial systems can be designed, refined, and optimized prior to synthetic efforts, in an efficient manner. Moreover, computational approaches allow parametric exploration of parameters (e.g., interaction energies, extreme temperatures or pressures) difficult to attain experimentally, and can thus be used to develop and confirm theoretical descriptions beyond a finite set of empirical data points. This is one of the governing foundations of the recently proposed Materials Genome Project [348], removing guesswork from materials design and “*accelerating materials discovery through advanced scientific computing and innovative design tools.*” The concept that combinatorial computational screening can lead to material discovery and property optimization has been recently demonstrated in applications such as solar energy generation [349] and battery development [350]. These successes, however, typically involved relatively exotic metal alloys and compounds. For
biological and medical applications, physiological effects of the material such as toxicity must be considered, complicating matters. A more rational screening method must be applied, using known biocompatible materials as a platform, and considering system interactions beyond single property optimizations. This leads to the emerging field of materiomics.

‘Materiomics’ is defined as the systematic study of the complete material system and it examines links between physiochemical material properties and material characteristics and functions, a paradigm similar to systems biology [291, 296, 351-352]. Through materiomics, fundamental advances in our understanding of biological systems contribute to the mechanistic understanding of certain diseases and facilitate the development of novel biological, biologically inspired, and completely synthetic materials for applications in medicine (biomaterials), nanotechnology, and engineering. Inspired by genomics, many more ‘omic disciplines’ have been defined and studied (Table 7.2). ‘Omic’ is traditionally a general term for a broad discipline of science and engineering for analyzing the interactions of biological systems which can also be referred to as high-dimensional biology [353]. Such fields are typically characterized by general systems (such as genomics for genes or proteomics for proteins) or processes (e.g., transcriptomics for genetic transcription or interactomics for cellular interactions). Omics technologies offer a system-wide approach to discover novel diagnostics and biomarkers for personal health monitoring [354-356], while the new emerging field of materiomics forges innovative bridges between omics systems science and materials science [291, 351].

<table>
<thead>
<tr>
<th>Omics</th>
<th>Definition</th>
<th>Focus</th>
<th>Scope</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Omics</td>
<td>Informally referring to a field of study in biology ending in -omics</td>
<td>Analyzing the interactions of biological information in various 'omes'</td>
<td>Applying research paradigm to produce knowledge en masse from networks of information via holistic principles and methods</td>
<td>[353, 357]</td>
</tr>
<tr>
<td>Genomics</td>
<td>A discipline in genetics that applies recombinant DNA, RNA sequencing methods, and bioinformatics to sequence, assemble, and analyze the function and structure of genomes</td>
<td>An organism's entire hereditary information; <strong>genome</strong></td>
<td>Determination of entire DNA sequences of organisms, fine-scale genetic mapping including genes, regulatory and noncoding sequences</td>
<td>[353, 358]</td>
</tr>
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<td>------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>Proteomics</td>
<td>The study of proteomes and their functions</td>
<td>Protein characterization, protein-coding regions of the genome; <strong>proteome</strong></td>
<td>The entire complement of proteins produced by an organism or system, including protein structure, function, and expression</td>
<td>[353, 359-360]</td>
</tr>
<tr>
<td>Transcriptomics</td>
<td>The study of the transcriptome using high-throughput methods</td>
<td>RNA transcripts produced by the genome at any one time; <strong>transcriptome</strong></td>
<td>Examining the expression level of RNA in a given cell population, which vary with external environmental conditions, including mRNA, rRNA, tRNA, and noncoding RNA</td>
<td>[353, 361]</td>
</tr>
<tr>
<td>Materiomics</td>
<td>The holistic study of material systems, examining links between physiochemical material properties and material characteristics and function</td>
<td>Material characterization through components, structure, and function; <strong>materiome</strong></td>
<td>Analysis of material systems through constitutive components, hierarchical SPP relations, cross-scale interactions, and effects on functionality</td>
<td>[291, 351]</td>
</tr>
</tbody>
</table>

*Citations provided are representative examples and not intended to be canonical works.

Materiomics can be conceptualized as the convergence of materials science, biological science, and technological advancements (encompassing computational methods and experimental tests) (Figure 7.11). It provides a complete toolset required to describe the complexities introduced by multi-scale relations, discrete hierarchical materials, cross-phase interactions, and structure-property dependencies across all scales which are critical to all biological materials. Moreover, materiomics can be applied in elucidating the biological role of materials, for example, in the diagnosis of diseases or even be exploited to predict diseases in the context of diagnostic tools by measuring material properties rather than focusing on symptomatic chemical readings alone, providing new strategies for treatment options. It can also be utilized to develop novel biomaterials which possess great diversity of potential in fields of bioengineering, biomedical devices and medicine. Inevitably, materiomics directs the advancement of nanoscience and nanotechnology, where materials science from biology will enable the bottom-up development of unique and effective biomaterials and devices.
Fig. 7.11. Scope and effect of Materiomics. The interface of materials science ("synthetic") and biology ("life") has been successful in the development of new biomaterials, but recent technological advancements ("technology" - computational capabilities, experimental methods such as AFM, and imaging techniques such as NMR) allow for a truly integrated and holistic approach. Image reproduced with permission from Cranford et al [291].

It is stressed that the umbrella term “materiomics” is not an attempt to introduce a new field of science. Rather, it is a unifying proposition motivated by the convergence of many fields towards a fundamental integrated description of materials and their functional roles. Materiomics takes a materials science perspective towards complex biological systems, explicitly accounting for feedback loops that link functional requirements (and changes thereof) to altered material components and structures, at different scales in both time and length. It overcomes the barrier that currently separates the understanding at different length and time-scales, through the development of new experimental synthesis and characterization methods, novel model systems and an enhanced appreciation for a multi-scale view of materials in general, in order to fully understand multi-scale
or cross-scale interactions of the materiome [352]. To fully explore the potential of materiomics, systematic development of general methodologies is needed that are applicable to a number of material systems, living and synthetic. This asks for development of new tools and insights in the design of (evolutionary) search algorithms, performance metrics, and universal characteristics across systems. The ultimate goal of materiomics is to reduce the complexity of biosystems and to enable effective engineering solutions to shape tomorrow’s technology and innovation.

A clear application at the interface of biology and materials lies in oral health monitoring. One’s breath, for example, has a vast amount of data that can be extracted with the right diagnostic tool. Similar to an automobile’s catalytic system for exhaust, one can potential design a material platform to screen a patient’s breath for a tangible breadth of biomarkers. Beyond general health monitoring, determination of infectious oral diseases, caries, gingivitis, and periodontitis using bioinformatics has also attracted a great amount of attention [362-363]. For example, Gürsoy and their group used an in silico gene/protein interaction network model to define inflammatory proteins in saliva, induced or inhibited by estradiol, as early diagnostic biomarkers or target proteins related to pregnancy-associated gingivitis [362]. Keskin et al. have concluded that human beta defensing (hBD)-2, can be employed as a diagnostic and therapeutic tool to improve the quality of life of susceptible individuals and minimize the economic costs of the two major global public health problems – Crohn’s disease and Periodontitis [363]. They also proposed that a unique angle pertinent for both diagnostic and therapeutic sciences involves rethinking clinically distinct diseases with a view to their shared molecular targets, interactions, and pathophysiologies. Materiomics plays a major role in assessing the interactions between biomaterials and targeted biomarkers. While we focus on a specific application (i.e., DNA-based detection), the design methodology – namely optimizing molecular building blocks based on known performance metrics – can be easily adapted for other platforms, pathologies and diagnosis. Again, building on concepts from the Materials Genome, it is the ultimate goal to develop a contained computational “black
box” approach to integrated materials system design, optimizing materials properties and behavior

in silico, and enabling rapid development of clinical applications.
CHAPTER 8

CONCLUSIONS AND FURTHER RESEARCH DIRECTIONS

Point of care (POC) diagnostics and monitoring attracts more and more attention in healthcare industry and plays a very important role in everyday life of many individuals. Our goal is to clinically monitor a person’s health status, disease progress, and treatment results through the use of an integrated and combined saliva and breath sensing system based on nanotechnologies. It is to be noninvasive, easy to collect the sample without fear or pain, fast in response, portable and reusable with disposable strips that are affordable. Its impact is a device to be used at home, doctor’s office, and medical clinics as the first line of defense to treat diseases and for worldwide adoption. It is not to replace the very expensive stationary equipment using blood and very sophisticated equipment like MRI and biopsy procedure for final diagnosis of the disease; but it is intended to become a home care device to monitor and diagnosis the onset of a disease such as diabetes, oral and lung cancers, and heart disease, just to name a few. Such a low-cost device, compared to
expensive laboratory equipment, would also have great benefits for use in remote areas and
developing countries. The key is using biomarkers from saliva to scrutinize an onset of a disease
and its stages, and chemicals or biomarkers from a puff of the breath can be used to monitor and
warn a specific disease at early stage. Our proposed technology platform is to use these biomarkers
and chemicals in saliva and breath to rapidly diagnose a disease and monitor its progress.

To detect biomarkers in saliva, we developed an electrochemical saliva sensing platform
consisting working, counter, and reference electrodes through micro-fabrication. Applying a layer-
by-layer (LBL) assembly of SWNT and multilayer films composed of chitosan (CS)-gold
nanoparticles (GNp)-glucose oxidase (GOx), we have developed a unique, disposable glucose
nano-biosensor to provide accurate, low cost, and continuous glucose monitoring through saliva.
More than eight clinical trials on real-time noninvasive salivary glucose monitoring were carried
out on two healthy individuals (a 2-3 hour-period for each trial, including both regular food and
standard glucose beverage intake with more than 35 saliva samples obtained), and excellent clinical
accuracy was revealed as compared to the UV spectrophotometer. The limitation of this study is
that only healthy individuals were included so that there were no extreme high/low glucose values
detected. The study of the relationship between blood glucose and salivary glucose based on age,
gender, medical, and health conditions is highly needed. These outcomes would improve statistical
confidence by increasing the study population.

We also found that it is very promising to use electrochemical sensors to detect protein
biomarkers from saliva. With the right components, this easy, fast and cost effective detection
method can be very powerful. Interest in saliva even more increased with the finding that saliva is
filled with hundreds of components that can serve to detect systemic disease or evidence of
exposure to various harmful substances, as well as provide biomarkers of health and disease status
[364]. Many salivary proteins offer great potential in clinical and epidemiological research, in oral
as well as in general health monitoring [365].
Furthermore, we have developed a wireless sensor array based on single-stranded DNA (ssDNA)-decorated single-walled carbon nanotubes (SWNT) for the detection of some physiological biomarkers in breath. Four different DNA sequences were used to functionalize SWNT sensors to detect trace amount of methanol, benzene, acetonitrile, dimethyl sulfide, hydrogen sulfide, and acetone, which are indicators of heavy smoking, excessive drinking, and disease such as lung cancer and diabetes. Our tests indicated that DNA functionalized SWNT sensors exhibit great selectivity, sensitivity, reproducibility, and repeatability. Different molecules can be distinguished through pattern recognition enabled by this sensor array.

We also developed a DNA screening method via full atomistic steered molecular dynamics simulation to select the best DNA sequences to react with chemicals useful in disease diagnosis and monitoring. Future work will focus on 1) applying full atomistic molecular dynamics characterization approaches to a subset of DNA sequences and more selected biomarkers, 2) developing an automated optimization process rather than manually assess all possible sequence variations in a brute-force approach, and 3) conducting experiments for validation. We intended to exploit DNA as a tunable material for sensing applications by using computational screening and in future a novel sequence exchange approach. Later on, this systematic design methodology can be applied to tailor the behavior of a material system driven by molecular interaction metrics.

The future work would be to combine these two separate saliva and breath sensing system into a technology platform which can detect multiplex biomarkers at the same time. This combined process can very effectively reduce the false positive cases in the disease diagnosis or screening by comprehensively monitoring biomarkers in saliva and breath. Thus, it will be able to rapidly diagnose a disease and monitor its progress and good for general health monitoring as well.
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