Upgraded resolution and enhanced sensitivity of optical disc-based bioassays: from CD to BD

by

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Abstract

Conventional biomedical diagnosis and chemical analysis are performed by trained professionals in centralized laboratories using specialized instrumentation. Our group has been working on the development of optical disc technology-based molecular detection platforms for on-site chemical analysis and point-of-care diagnosis for the past 15 years. In particular, computer-readable assays on compact discs (CDs) have been demonstrated for the quantitation of various target analytes, such as DNA, proteins, and metal ions. In this thesis, two more advanced disc formats, digital versatile disc (DVD) and Blu-ray disc (BD) have been adapted to improve the assay resolution and detection sensitivity. The DVD assay for quantitative analysis of human chorionic gonadotropin is described first. A multiplex assay-on-a-BD system for the detection of a set of key cardiac markers is then explained in detail. Beyond a different error correction protocol, a new surface activation method has been established for the immobilization of probe molecules on the surface coating of BD-Rs. In addition to the upgraded lateral resolution, the sensitivity of the BD-based bioassays is significantly improved in comparison with those prepared on CDs and DVDs, which is comparable to the well-established enzyme-linked immunosorbent assay (ELISA).

Keywords: Digital versatile discs (DVD); Blu-ray disc (BD); point-of-care (POC) diagnosis; human chorionic gonadotropin (hCG); pregnancy test; cardiac marker; acute myocardial infarction (AMI)

To my family and friends

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List of Acronyms

AMI	Acute myocardial infarction
BD	Blu-ray disc
BIS	Burst indicator subcode
BSA	Bovine serum albumin
CD	Compact disc
CIRC	Cross-Interleaved Reed-Solomon Code
CRP	C-creative protein
cTnI	Cardiac troponin I
cTnT	Cardiac troponin T
DVD	Digital versatile disc
ECG	Electrocardiographic
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDS	Energy-dispersive X-ray spectroscopy
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle-stimulating hormone
hCG	Human chorionic gonadotropin
HRP	Horseradish peroxidase
LDC	Long distance code
LED	Light-emitting diode
Mab	Monoclonal antibody
MB	Myoglobin
MES	N-morpholinoethane sulfonic acid
NHS	*N*-hydroxysuccinimide
ODR	Optical darkness ratio
PDMS	Polydimethylsiloxane
PIE	Parity inner errors
PIF	Parity inner failures
PMMA	Poly(methylmethacrylate)
POC	Point-of-care
SAM	Self-assembled monolayer
SEM	Scanning electron microscopy

SERS	Surface-enhanced Raman spectroscopy
SPR	Surface plasmon resonance
TMB	3,3',5,5'-tetramethybenzidine
TSH	Thyroid-stimulating hormone

Chapter 1.

Introduction

1.1. General introduction: from disk to disc

The words “disk” and “disc” are commonly used to describe a thin and circular object, but many people are not aware of (or agree on) the difference between the two. In computing science, a “disk” refers to any non-optical storage medium whereas a “disc” refers specifically to an optical storage device such as a compact disc (CD), a digital versatile disc (DVD), or a Blu-ray disc (BD).[1] In this chapter, a brief description of the development of microfluidic “lab-on-a-disk” devices, interferometric disks, and other specially fabricated analog disks for optical detection will be presented first. Although these analog “disks” are not exactly related to the optical disc technology (as they do not bear functions for optical data storage), they are relevant to the thesis topic and have been well studied in the past two decades. I will then introduce the fundamentals of optical disc technology (e.g., disc construction and recording principle) and highlight the differences between the disc types (CD, DVD, and BD). It will be followed by a comprehensive summary of the past and present research on the development of analytical devices by directly adapting optical disc and recording/reading technology, i.e., by modifying optical drives and disc players, “derivatizing” disc media, and exploring software protocols (using *bona fide* optical discs and computer drives).

1.1.1. Microfluidic lab-on-a-disk (LOD) devices

Traditional quantitative analysis is usually time-consuming and requires strict working conditions such as trained professionals and special equipment. Laboratory or hospital test results can take several days to weeks to obtain the results, causing delayed treatments for patients.[2] Consequently, over the past 30 years, the development of lab-

on-a-disk (LOD) devices has been a major focus in both industrial and academic research, leading to its emergence as an advanced point-of-care (POC) diagnostics device.[3,4] These LOD systems take the shape of a CD and exploit a combination of capillary and centrifugal forces to drive the liquid flow.[3,5] A simple motor may be required to trigger the analysis; centrifugal forces induced in sample fluids enable the radial movement of fluids from the inside to the outside of the disk. Based on this concept, auto-manipulation of liquid samples can be achieved with specially-fabricated microfluidic disks.

In the early stages of microfluidic platform development, microarrays were created on a glass, plastic, or gold chip by attaching two polydimethylsiloxane (PDMS) microchannel plates consecutively in a perpendicular orientation to generate microarrays at the intersections.[6,7] An external syringe pump was usually required to overcome insufficient delivery of liquid solutions by only capillary action. In the early 2008, Peng *et al*. took advantage of centrifugal forces to perform DNA hybridization on a CD-like glass chip without an external pump.[8] Testing of a 96 × 96 hybridization assay was carried out in a two-step assembly process. Figure 1.1 shows the designs of CD-shaped PDMS chips. A chip with 96 radial channels was used for the first step (DNA probe immobilization), and a second PDMS chip with 96 spiral channels were used for the second step (the hybridization event occurring at the intersection of the spiral and radial channels). The solution delivery in the channels is driven by spinning capillary force in both steps. The method was further developed to build detection platforms for plant fungal pathogens.[9]

Independently, Honda *et al.* used a commercially available Gyrolab CD, a CD-shaped polymer plate with an intricate microfluidic network, to automate multiple sandwich immunoassays.[10] The microfluidic channels are separated by hydrophobic barriers to control the liquid flow to a specific volume. When the volume definition chamber is completely filled, the excess liquid flows to an outflow reservoir. This disk design is beneficial for controlling the sample/reagent volume and for shortening the total dispensing time.

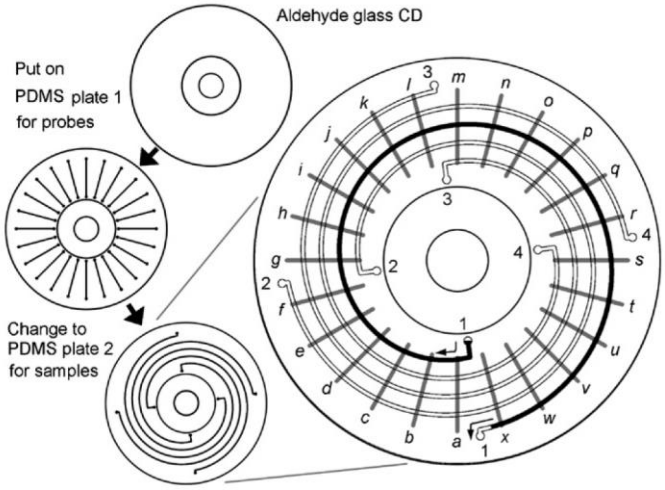

Figure 1.1. Schematic view of the microfluidic microarray chips. The PDMS chip 1 was used for immobilization of DNA probes (solid radial lines marked as a-x). PDMS plate 2 was used for four samples (hollow spiral lines marked by 1-4). The black line and the two arrows indicate the liquid flow in the channel 1. Reprinted with permission from reference 8. Copyright © 2007, Elsevier.

Recently, more lab-on-a-disk devices have focused on full integration and automation in blood separation, metering, aliquoting, mixing, reagent storage, and sequential reagent delivery,[3] to name a few. To achieve these fluid manipulations, the evolution of valving technology played an important role. For example, in 2012 Park *et al*. reported an active valving mechanism utilizing laser irradiation to actuate and melt ferrowax microvalves to accomplish sequential fluid transfer and delivery while avoiding surface energy changes associated with passive valving.[11] They also presented a fully integrated multiplex immunoassay for detection of cardiovascular disease biomarkers from saliva and serum samples. Figure 1.2 shows the disk design and working principle of the laser-induced valving technique. The corresponding quantitative analysis results depend on absorbance measurements at the photodiode and light-emitting diode (LED). In their work, no user intervention is required throughout the entire detection process except for the initial loading of the reagents.

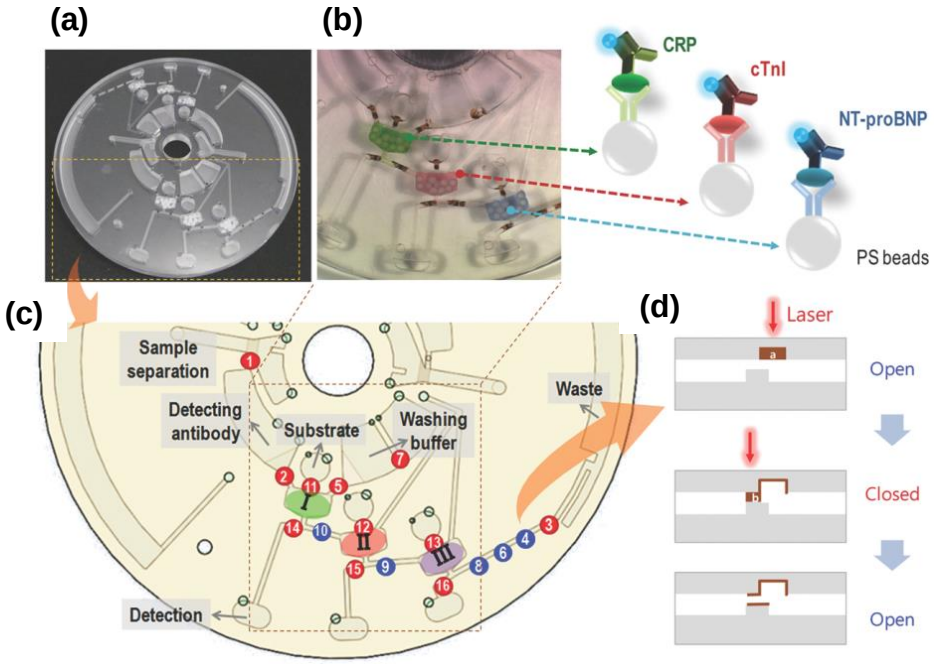

Figure 1.2. Optical images of (a) fabricated disk for detection of cardiovascular disease biomarkers; (b) the reaction chambers preloaded with functionalized polystyrene beads. (c) The detailed microfluidic disk design. The blue and the red circles indicate the state (opened and closed, respectively) of laser irradiated ferrowax microvalves and the number indicates the order of the valve operation. (d) A schematic diagram of laser irradiated ferrowax microvalve operation. The laser induces the valve opening and closing position. Reprinted with permission from reference 11. Copyright © 2007, American Chemical Society.

Meanwhile, Nwankire *et al*. demonstrated another valving concept based on water-soluble films to accomplish a fully integrated portable multiplexed centrifugal microfluidic disk for liver function screening.[12] Their unique dissolvable-film-based centrifugo-pneumatic valves provide advanced flow control for plasma extraction, metering, and aliquoting into separate reaction chambers for colorimetric quantification during rotation. The portable and multiplex disk is sandwiched between the LED emitter and detector for optical read-out. The whole process can be done within 20 minutes while only a tenth of the reagent volume is required compare to standard hospital laboratory tests.

Lab-on-a-disk technology not only involves the design of microfabricated disks as detailed above, but also requires the configuration of integrated systems that incorporate

optical, electrochemical, or mechanical signal readout protocols for the identification and quantitation of biomolecules.[13] The three major reasons for the growing popularity of disk-based analytical platforms are the reduced sample and reagent consumption, convenient fluid delivery along the disk by centrifugal force, and potential for rapid detection.[14] These benefits of LOD tools make them suitable candidates for POC diagnosis, which may be adapted to a broad range of biomedical applications, such as gene profiling and standard immunoassays.[3,5,15]

1.1.2. Analog disks specially fabricated for optical sensing

Differing from LOD devices, the so-called "bioCD",[16] was pioneered by Nolte and co-workers are custom-made analog disks for interferometric detection of biomolecular analytes. These disks were prepared on a CD-shaped silicon wafer or laser mirror with nanostructured gold radial spokes on top. By monitoring the phase and intensity changes at quadrature upon immunobinding, one can measure the quantity of bound analyte without labelling. The performance of the bioCD approach has been evaluated by the measurement of prostate specific antigen on 96-well and 25,000-spot disks.[17,18]

An ingenious strategy to construct bioDVDs was introduced by Gopinath *et al*. in 2008.[19,20] The conceptually different bioDVD has a specially constructed five-layer structure and was employed as a label-free detection platform for nucleic acid hybridization and RNA ligand/protein binding. Their fabrication of bioDVD with an optimal thickness of the multi-layered metal/dielectric film was accomplished by radio frequency magnetron sputtering on a pre-grooved polycarbonate disk. The quantification is based on the change in reflected light intensity due to analyte adsorption to the sensor surface.

As described above, it can be concluded that the development of disk-based analytical platforms (including LOD devices) has been a very active area in academic research. However, there are challenges in the sample preparation steps for multiplex assays. The automation of sample handling can simplify the sample preparation procedure, but at the same time increase the cost and complexity, which limits its application in POC settings. Challenges in long-term reagent storage on the disk also need to be overcome.[3] In fact, disk-based analytical platforms have attracted several companies

in the current market to focus on the development of spinning disk diagnostic platforms and attempt to widen their applications. For instance, Gyros AB (Uppsala, Sweden) produces a CD-like microfluidic platform, the Gyrolab CD, designed for automated sandwich immunoassay processing for high-throughput analysis. Quadraspec (Indiana, USA) developed spinning disk interferometry that provides a label-free detection system to identify changes in protein arrays, while Burstein Technologies (California, USA) developed special microarray disks and stand-alone readers for forensic DNA analysis, as well as for food and water contamination testings.[15]

1.2. Optical discs and recording/reading technology

Apart from the microfluidic lab-on-disks and interferometric bioCDs, our focus will be the development of analytical tools that are directly related to optical discs and recording technology. Before going into the details of the new research advances, it is necessary to provide a general introduction to various disc structures and their respective recording/reading mechanisms.

1.2.1. Structure and recording principle of optical discs

CDs were first introduced in Japan in 1982.[21] Since then, optical disc technology has been continuously improved and advanced, leading to the development of DVDs and BDs. Optical discs (CD, DVD, and BD) have been used popularly for storing digital data and media files in the past 30 years. A standard optical disc has a diameter of 120 mm and a center hole (15 mm in diameter) with a thickness of 1.2 mm.[22,23] In the disc, the lead-in and lead-out areas, which are located at radii of 24 mm and 58 mm, respectively, guide the disc reader (optical drive) to recognize the start and end point of the data area.[1,22,23] A schematic diagram of optical disc structures is shown in Figure 1.3. All optical discs are comprised of polycarbonate substrates with tracks of “pits” and “lands”, which are created from a dye layer (data recording layer) or a molding process for the recordable/rewriteable and read-only memory media, respectively (*vide infra*). The track is covered by a reflective metallic layer (aluminum, gold, or silver) with a thickness of 50 to 100 nm and in some cases an additional layer of polycarbonate. A thin lacquer film is added last as a protective layer to prevent scratches on the disc during everyday use.[22,23]

Within the original types (read-only memories, ROM) of optical discs (CD-ROM, DVD-ROM) created from a molding process (no recording layer in these discs; the information is permanently stamped on the polycarbonate substrate), the heights of the pits are chosen to be exactly 1/4 of the laser wavelength (λ) such that light travelling to a land is delayed by 1/2λ of that travels to the pit next to it. The reflected light is exactly out-of-phase with that being reflected from the pit; therefore, no light can reach the photodiode due to the destructive interference, and the land-to-pit (or *vice versa*) transition is signified as a binary signal change from "1" to "0" (or *vice versa*). On the other hand, a laser beam reflected from a pit or land without a transition is constructive and no binary signal change is recorded.[23]

The density of pits and lands in the disc is increased from CD to BD that improves the storage capacity (Figure 1.4).[24] As the data layer on BD is much closer to the reading surface (as compared to CD and DVD), it provides more precision for data recording/reading since the laser beam travels a shorter distance in the polycarbonate layer (0.1 mm), resulting in less distortion from the refraction.

The recordable formats of optical discs (CD-R, DVD-R, and BD-R) have the same basic structure, but with significant detail differences (Figure 1.3). A recordable disc has a dye-based data-recording layer, with a reflectivity of 45 - 75 %,[1] located beneath the reflecting metal layer and above the polycarbonate substrate. These discs have a track spiral that is pre-formed during manufacture, onto which the data is written during the recording process. This track ensures that the recorder follows the same spiral pattern as conventional optical discs.

In conjunction with optical discs, optical drives have been the standard equipment on personal computers for writing and reading digital information to and from discs. The typical components in an optical drive include a photodetector, a laser diode, focusing lenses, a beam splitter, and a servo motor. The first three parts are the main components of an optical pickup unit.[1] The bottom inset of Figure 1.3 illustrates the working principle of an optical readout system. In brief, during the reading process, the diode laser is directed by a prism (beam splitter), which rotates the plane of polarization of beams. The light beam is then collimated and produces a parallel beam by a collimator. This parallel

beam is focused onto the data track in the disc after passing through an objective lens. The laser beam scans the surface of the disc in a spiral pattern from the inner circle to the outer one as the disc rotates counter-clockwise. The reflected light from the disc surface is then split into two by a prism. This prism serves as an output coupler that only allows light to pass through in one plane to reach a detector (photodiode). The photodetector signals are then decoded by a microprocessor and finally recognized by a computer in the form of music, movie, or other digital data. In fact, the evolution of optical disc technology not only involves changes in the disc structure, but also in the optical drives (Table 1.1).[23,25] The most significant improvement is the laser: CD drives utilize a near-infrared laser (780 nm), DVD drives utilize a red laser (650 nm), and BD drives utilize a violet laser (405 nm).[23,25] The diameter of the laser focus spot is proportional to λ/NA where NA is the numerical aperture of the objective lens.[26] NA of objective lens is defined by the following equation

$$\mathrm{NA} = n \sin \theta \tag{1}$$

where n is the refraction index of the medium and θ is the maximum angle of incidence. Compared to CD and DVD drives, BD drives utilize a laser of shorter wavelength and objective lenses with larger numerical apertures that effectively decreases the focus size of the laser to 0.64 μm, thereby providing better lateral resolution (high capacity) for data recording and reading. The diameter of the laser spot at the polycarbonate surface is 800, 500 and 130 μm for CDs, DVDs and BD, respectively.[27] Since BDs are more sensitive than DVDs and CDs to damages from slight scratches or fingerprints (which may cause deterioration of error rates and/or loss of a tracking servo control), a hard coating layer (e.g., a colloidal silica-dispersed UV-curable acrylic, methacrylic, vinyl or styrene resin, or a methacrylate-based lactone material) is introduced for surface protection.[22,26]

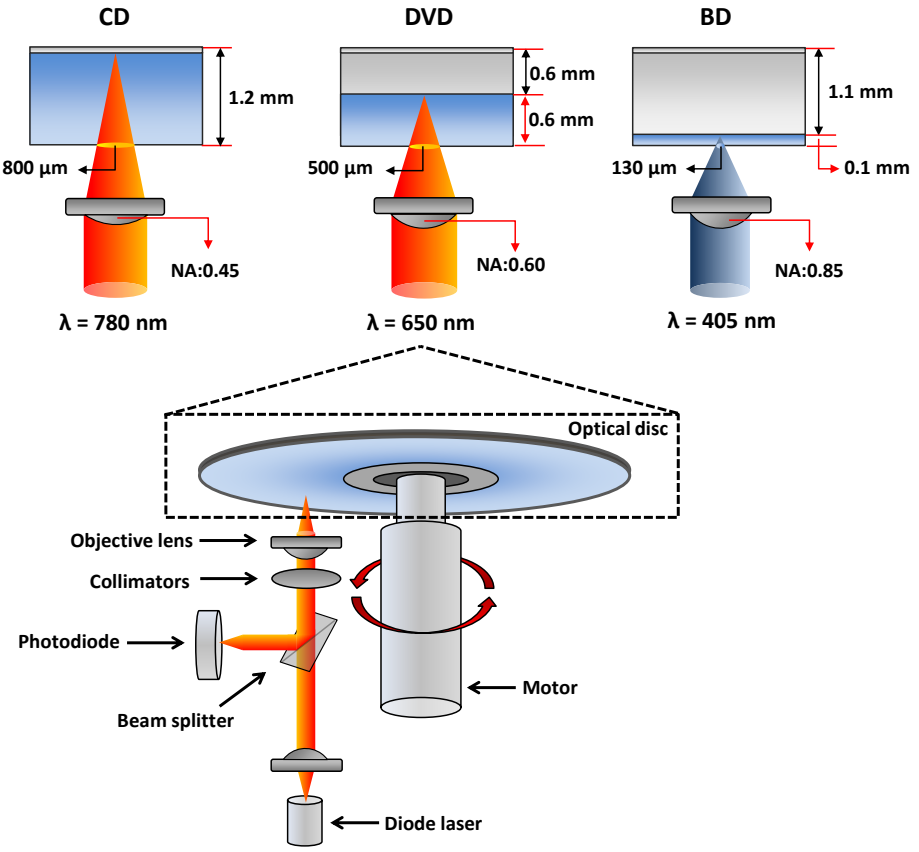

Figure 1.3. **Schematic view of optical disc structure (simplified) and reading mechanics. The laser beam travels from the laser diode towards the metallic layer in the disc, the reflected light is detected by the photodiode.**[22,28]

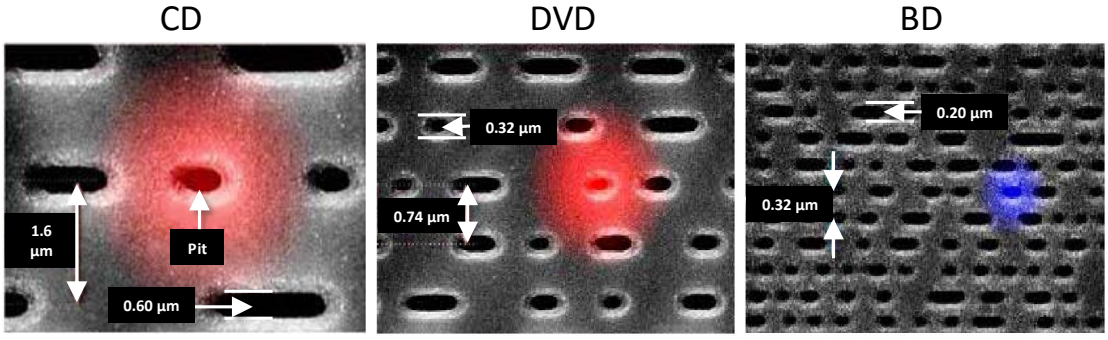

Figure 1.4. **SEM images showing the different pit dimensions in CD-ROM, DVD-ROM, and BD-ROM. The distance between the tracks decreases as the disc evolves from CD to BD. Adapted with permission from reference 24. Copyright © 2010, Blu-ray Disc Association.**

Table 1.1. Comparison of optical discs and their key parameters[23,25]

Disc format	CD	DVD	BD
Capacity (gigabyte, GB)	0.65	4.7	25
Laser wavelength (λ, nm)	780	650	405
NA	0.45	0.60	0.85
Diameter of the laser focus spot (μm)	3.2	1.48	0.64
Substrate thickness (mm)	1.2	0.6	0.1

Note: NA = numerical aperture of the objective lens

1.2.2. Data structure and error correction mechanism

Discs for audio and video recording typically implement an error correction mechanism to ensure that even when the discs are slightly damaged (e.g., scratched), the data can still be recognized. As the error correction mechanism has been exploited as the digital signal readout system in this thesis (*vide infra*), it is therefore important to understand the error types, the correction mechanism, and data structure in the different types of optical discs.

In general, there are two types of errors, single errors (or random errors) and burst errors. Single errors only affect one or two bytes of data and are typically distributed randomly in the data. These errors are usually caused by manufacturing defects or signal deterioration such as noises from tilting of the disc or defocus of the laser. Burst errors on the other hand are produced by scratches, dusts, or fingerprints on the disc surface.[26,29]

In optical disc technology, Reed-Solomon (RS) codes are linear block-based error correcting codes commonly used in digital storage (CDs, DVDs, and BDs) for error correction. Message symbols are coded as blocks, and extra parity symbols are added to each block by the RS decoder. In brief, a RS code is denoted as RS(n,k) with s-bit

symbols. For *k* data symbols of *s* bits each, parity symbols are added to make a total length of *n*. RS codes are able to correct up to *t* errors in a code word where $2t = n-k$ (Figure 1.5). For example, if there is a RS (255, 223) with 8-bit symbols, it means that n, k, and s are 255, 223, and 8 respectively. In this case, the decoder can correct up to 16 symbol errors in the code word.[30,31]

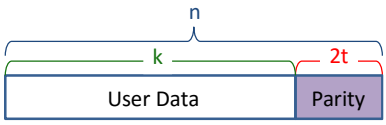

Figure 1.5. Representation of Reed-Solomon codes. The total codewords (n) consist of k data symbols of s bits each and 2t parity symbols. t is the maximum number of symbol errors that can be corrected. Adapted from reference 30.

Cross-Interleaved Reed-Solomon Code (CIRC) is often used by disc drives for error correction detected from the discs. By interleaving the RS codes, a large portion of errors can be distributed into much smaller parts throughout the disc that minimizes the loss of recorded information and thereby increases the chance of correcting the errors. Figure 1.6b shows a schematic representation of the cross-interleaving result.

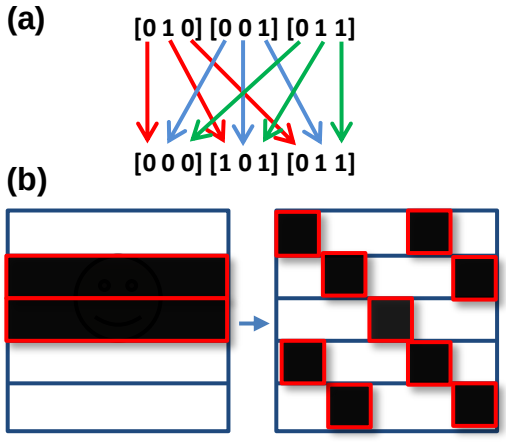

Figure 1.6. Schematic representation of the CIRC working principle. (a) The interleaving pattern of the data. (b) Schematic representation of the interleaving results. The data remain uninterrupted and are readable after being interleaved. Adapted from reference 31.

In the CD system, data are divided into blocks of 2352 bytes. Each of the blocks is composed of 98 identical frames. Within these frames, there are 3 bytes of synchronization, 1 byte of subcode, two interleaved audio data (12 bytes each), and two parity (4 bytes each).[32,33] Figure 1.7 shows the general data structure of an audio CD. Since 75 blocks are read per second from a CD at normal speed, a full 74-minute disc contains 333000 blocks. There are two types of error correction in a CD system: C1 and C2. C1 is designed to correct random errors and detect burst errors, while C2 is designed for the correction of both burst error and random errors that are not corrected or miscorrected by C1. C1 corrects only one random error per frame, but C2 can correct up to four errors per frame.[34]

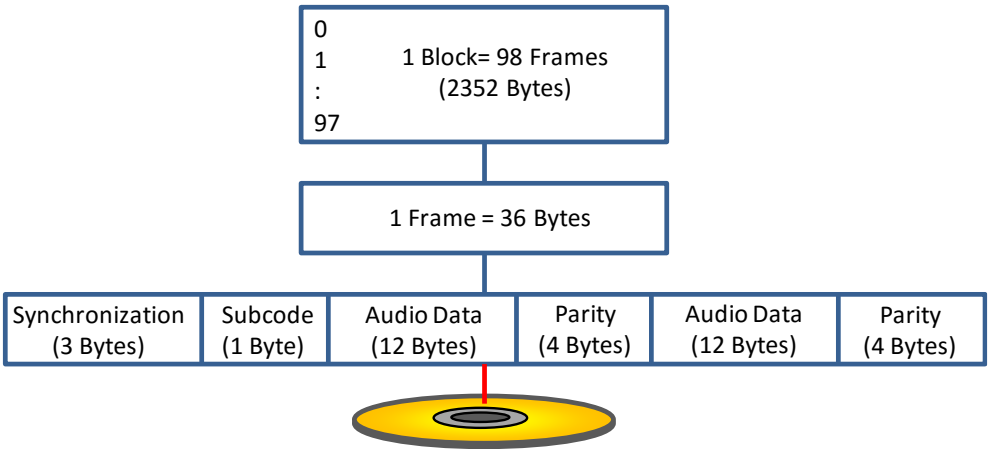

Figure 1.7. CD data structure. One block has 2352 bytes consisting of synchronization, subcode, audio data, and parity. Inspired by reference 33.

In a standard DVD, an error correction code (ECC) block consists of 16 data sectors (Figure 1.8a), 16 rows of outer parity (PO) and 10 bytes of inner parity (PI) data in each row, forming a total of 32768 bytes (Figure 1.8b). Each data sector has 2064 bytes in total where 2048 bytes are user data and 16 bytes are logic and error detection codes. Specifically, within these 16 bytes, 4 bytes belong to identification data (ID), which indicates the address of the DVD sector, 2 bytes are used for indicating an error detection code of ID data (IED), 6 bytes contain copyright management information (CPR_MAI), and finally 4 bytes of error detection code (EDC) at the end of the sector.[35] The increase of PO size in an ECC block is possible to improve the correctable burst error length. However, the encoding efficiency of data information drops as the PO size increases due

to the increase of redundancy. The current DVD has an encoding efficiency of 87%. In a DVD, a logical ECC block is a basic unit when it undergoes disc quality testing. Disc quality can be expressed in terms of the numbers of parity inner errors (PIE) or parity inner failures (PIF). The fewer the numbers of PIE and PIF, the better the disc quality. When a row in an ECC block contains at least one byte of error, it generates a PIE. A PIF is generated when there are more than five bytes of error in a row of an ECC block.[35]

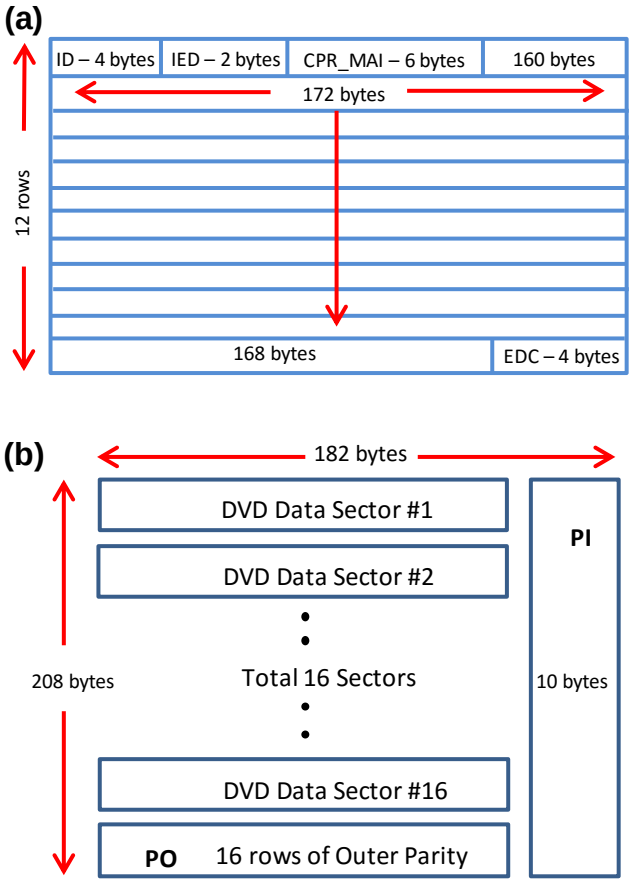

Figure 1.8. **DVD data structure and error detection algorism. (a) DVD Data sector (2064 bytes, 12 rows × 172 columns of data in total) that is composed of 2048 bytes of user data and 16 bytes of logic and error detection code. (b) A DVD ECC block has a total of 32768 bytes. It consists of 16 data sectors, 16 rows of PO and 10 bytes of PI per PO row. Adapted with permission from reference 35.**

In comparison, the data structure of a BD is significantly improved from that of a CD and a DVD. A BD ECC cluster consists of long distance code (LDC), synchronization (SYNC) code, and burst indicator subcode (BIS) with dimensions of 155 bytes × 496 bytes

(Figure 1.9). The LDC cluster is formed by user data and ECC parity with a total of 304 columns and 496 rows. This LDC cluster is re-arranged into LDC block and then evenly separated by a SYNC code column and three BIS (or picket code). The code words in a LDC block are interleaved two by two in the vertical direction, so that a block consisting of 152 bytes × 496 bytes is formed. A user data block within a LDC cluster is composed of 32 sectors, forming 65536 bytes in total. Each sector has 2052 bytes in total with 2048 bytes of user data and 4 bytes of EDC.[29] The left-most picket column is formed by the sync patterns at the start of each row. The other three pickets are protected by BIS that has 24 code words with 30 bytes of information symbols and 32 bytes of ECC parity symbols. These BIS code words are interleaved into three columns of 496 bytes each. Since a BD is very sensitive to small particles on the surface, the structure of the BD ECC cluster is improved by inserting picket columns between user data in order to provide a better error correction system. The locations of BIS code can be flagged as erasure during correction of code words for the main data. It should be noted that only one Reed-Solomon decoder is required to decode both LDC and BIS codes due to the identical number of ECC parity per code word.[29]

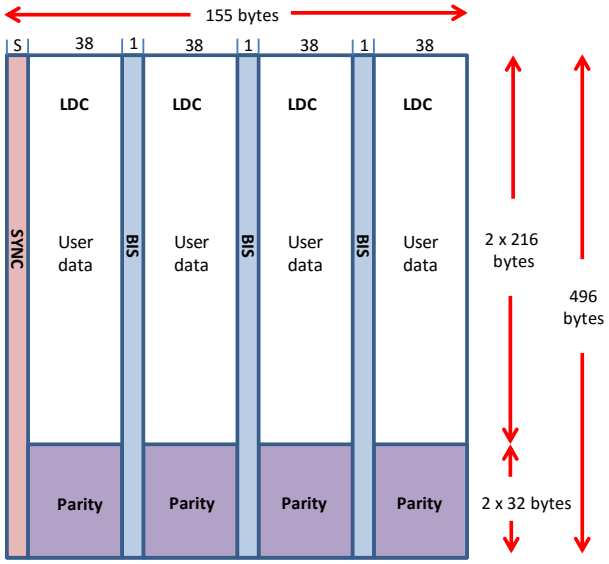

Figure 1.9. **BD data structure and error detection algorism. The BD ECC block has 65536 bytes in total. It consists of three main components: LDC, SYNC code, and BIS. Adapted with permission from reference 29. Copyright © 2004, Blu-ray Disc Association.**

1.3. Development of analytical devices based on optical discs and recording technology

Beyond their essential applications in portable data storage for the past 30 years, optical discs and corresponding recording technologies have been widely and extensively explored with the ultimate goal of creating integrated analytical systems for on-site chemical analysis and POC medical diagnosis. In particular, the disc media (CD, DVD, and BD) are proven to be inexpensive and versatile substrate materials for the preparation of various bioassays and microfluidic devices. In the meantime, conventional computer drives or disc players can be adapted as powerful optical detectors for biochip signal readout or optical imaging. This section provides an overview of disc technology-enabled analytical tools. The focus here will be systems that are created from specifically fabricated discs, modified optical drives, or adapted software protocols.

1.3.1. Modification of optical drives for sensing and imaging

Modification of optical drives either by altering the laser pickup unit or acquiring analog signals from the photodiode detector is one of the most popular approaches to create novel sensing or imaging devices for molecular analytes. In this section, I will describe various methodologies being developed to convert optical drives into optical readout or imaging systems. A common theme herein is the optical detection, i.e., detection based on the changes in optical properties (absorption, dispersion, and/or reflection) upon irradiation by the laser in the disc drives.[15,22]

In 2002, Alexandre *et al*. pioneered a doubled-sided CD reader consisting of two photodiodes to analyze a special “BioCD” for multiplex detection of hybridized DNA.[36] The first detector in this special doubled-sided reader is obtained from a conventional CD reader for retrieving the numeric information (inner section) on the Bio-CD. The numeric information provides a message to the first laser reader to control scanning position as well as rotation speed. The second detector is an additional laser photodiode mounted on top of the optical drive to gather genomic information (outer section) from the DNA microarray (Figure 1.10). The colored image of each array is created based on the amount of diffracted light detected by the photodiode. The quantitative analysis is accomplished

by measuring the relative grayscale intensity of each binding spot with respect to the background.

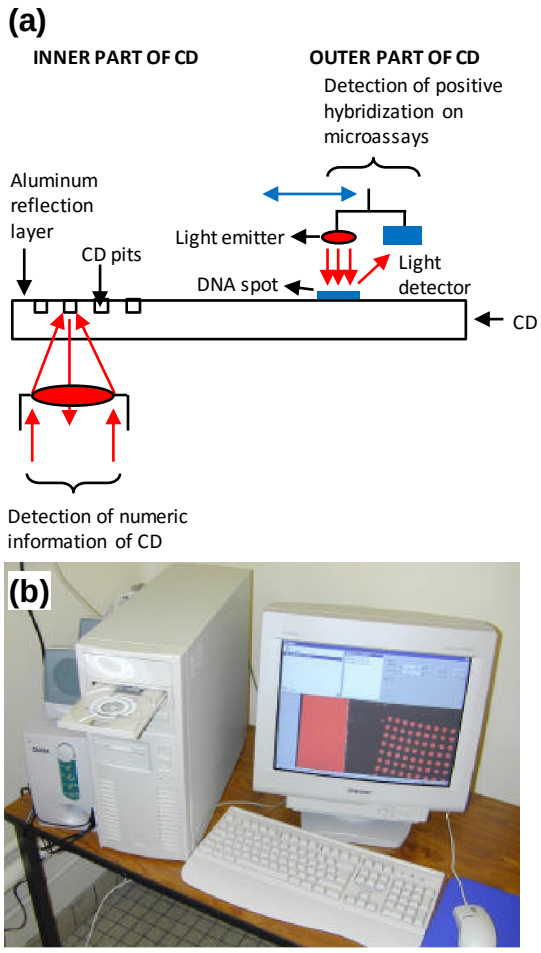

Figure 1.10. Doubled-sided CD reader for multiplex detection of hybridized DNA. (a) Schematic view of the reading system that is composed of two separated laser-based devices. One is for reading the numeric information located in the inner section of the CD and the other is for detecting the laser light diffraction from the DNA microarray. (b) Photo shows the experimental setup for colorimetric detection of DNA hybridization. Adapted with permission from reference 36. Copyright © 2002, BioTechniques.

Potyrailo *et al.* introduced a conceptually different strategy to adapt optical drives for molecular sensing.[37] Instead of installing an additional photodiode, they incorporated a customized data acquisition card to acquire analog signals directly from the photodiode in optical drives. The data acquisition card can be installed directly in a desktop or a laptop.

Figure 1.11 shows the setup, particularly the connections on the electrical circuit of the optical drive to obtain analog signals from the photodiode.

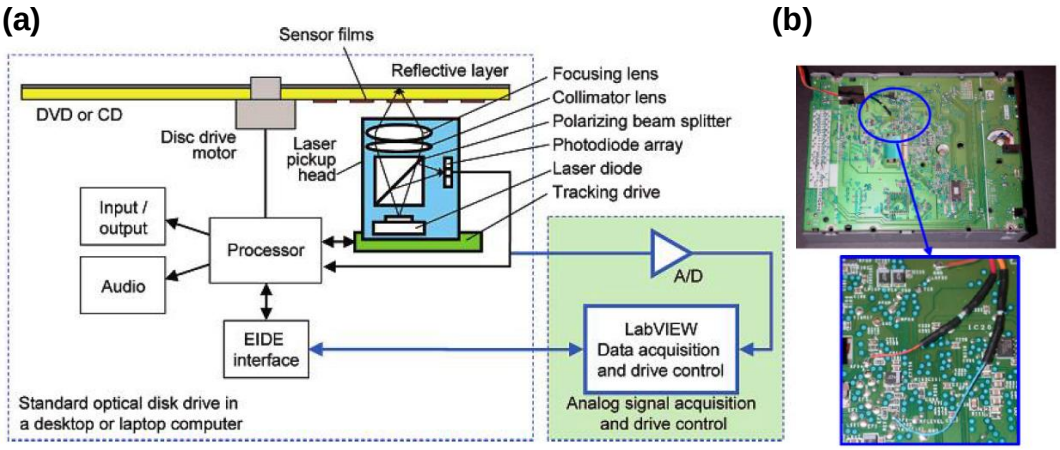

Figure 1.11. Concept for the Lab-on-a-DVD system based on analog signal acquisition. (a) Schematic diagram of a conventional optical drive and the methodology for obtaining an analog signal from a photodiode detector and for controlling the optical disc drive experimental setup. (b) Electrical connections to obtain analog signals from the optical photodiode component. Adapted with permission from reference 37. Copyright © 2006, American Chemical Society.

Potyrailo *et al.* also tried to minimize the extent of modification in the optical drive in order to maintain its original function of reading and writing digital content to optical discs, so that an ordinary optical disc can serve as a detection platform by simply exploiting colorimetric calcium-sensitive sensor films on top of the disc surface.[37] The corresponding DVD drive acquiring analog signals depends on the optical properties of the sensing films. The signal varies with the quantity of target species, which cause changes in the light intensity detected by the photodiode. The data acquisition program extracts these analog signals from the photodiode before the signals are digitalized during the reading process. In such a lab-on-a-DVD system, an enhanced integrated disc electronics (EIDE) interface controls the positioning of the laser pickup head for scanning the desired radial position and also the disc's rotation speed. They demonstrated the application of this platform for the quantitation of calcium ions in liquid samples based on analog signal reading with a detection limit of 5 ppm.

Recently, the use of DVD optical pickup unit in developing label-free sensing platforms has been reported by Bosco *et al*.[38] The optical pickup head from the commercially available DVD player is located 1 mm below the glass microfluidic disk. During the scanning process, the laser passes through the glass substrate and focuses on the cantilever surface. Bosco *et al.* utilized the optical pickup head to measure cantilever deflection, resonant frequency, and surface roughness. This platform was tested using detection of pesticide derivative 2,6-dichlorobenzamide.[38] Recently, Donolato *et al.* reported their first implementation of a Blu-ray optical pickup unit for the high-performance signal readout of a homogeneous assay in a multichamber microfluidic disc.[39] The assay detection relies on optical measurements related to the clustering dynamics of magnetic nanobeads in an applied oscillating magnetic field. A Blu-ray optical pickup unit was used for optical excitation and as a readout device. Laser light emitted from the optical pickup unit, which was controlled by a custom-made circuit, was transmitted through the sample chamber and reflected back to the photodiode of the optical pickup unit, allowing determination of the dynamics of functionalized magnetic nanobeads. The system is capable to detect *E. coli* DNA coil with a concentration in the 10 to 500 pM range.

Besides the application for optical signal readout in various chip-based devices as noted above, optical drives have been adapted for microscopic imaging. Lange *et al*. designed a scanning microscope (namely, the PickupImager), employing a standard CD/DVD pickup head that monitors the light reflection from silver-stained biomolecules on a disc for image acquisition.[40] In their work, the optical pickup unit in the CD/DVD player was removed from the optical drive and mounted on the stage of the scanning microscope to collect light reflected from the disc. A custom-built electronic board was used to control the laser focus position and amplify the measured signals. As shown in Figure 1.12, colloidal gold-labeled anti-biotin antibodies are recorded by their customized imager and its analyzed image is compared with those of both a standard optical microscope and an electron microscope before and after silver enhancement. This method is able to determine fractional surface coverage relative to the concentration of analytes. This analytical tool was tested with C-reactive protein with a detection limit of 1 pM.

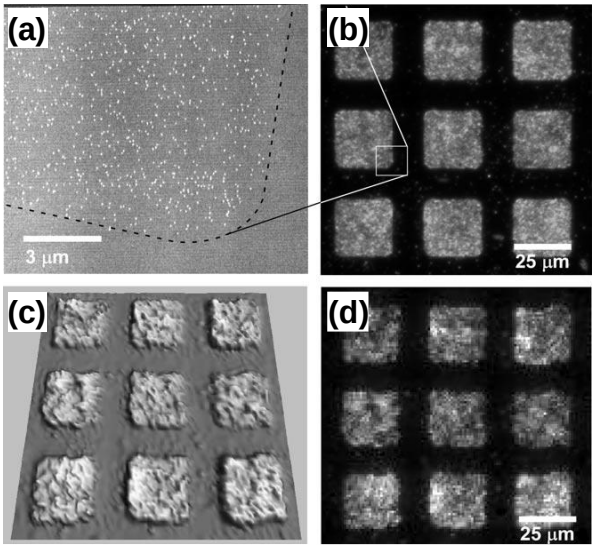

Figure 1.12. Imaging capability of the PickupImager. Microcontact-printed recognition assay pads (25 μm × 25 μm) were formed from colloidal-gold-labeled anti-biotin followed by silver development. (a) Scanning electron microscope image of the gold beads in the binding assay before amplification by silver enhancement. (b) Darkfield microscope image of the silver-enhanced array with a resolution of 0.3 μm. (c) and (d) are CD-pickup image of the same array of recognition pads after silver enhancement with a resolution of approximately 1 μm. (c) is an artificial 3D representation of the pickup data. Reprinted with permission from reference 40. Copyright © 2005, John Wiley and Sons.

In addition, Ramachandraiah *et al*. converted a commercially available DVD drive into a laser-scanning microscope (DVD-LSM) by incorporating an additional laser photodiode for an image-based diagnosis featuring bonded bioparticles (Figure 1.13).[41] The whole DVD-LSM system is composed of a standard DVD drive, thermocouples and an additional photodiode with a printed circuit board (Figure 1.13a). Figure 1.13c shows the differences between this DVD-LSM and an ordinary DVD drive. In the DVD-LSM, an additional photodiode was mounted on top of the disc surface to detect and record light transmitted through a semi-transparent DVD in order to produce particle images. A printed circuit board is designed to provide conversion of analog to digital signal of the captured data from the photodiode. As shown in Figure 1.13a, they have also modified a low-reflective multi-layered disposable polymer disc embedded in the U-shaped microfluidic channels as a detection platform. The inner walls of each channel are functionalized to specifically capture the targeted bioparticles. Ramachandraiah and his coworkers

demonstrated the potential medical use of this system for HIV diagnosis by counting CD4+ cells isolated from whole blood samples.[41] This technology has led to the development of a commercially available 'Discipher' platform by Ling Vitae (Husoysund, Norway).[15]

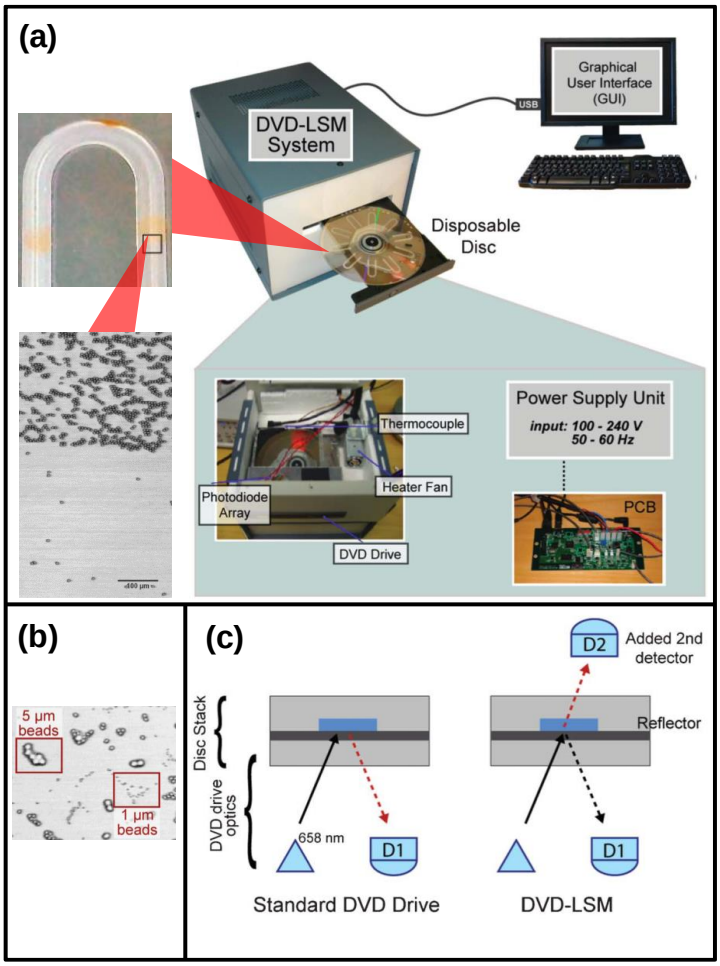

Figure 1.13. **Working principle and imaging capability of a DVD-LSM. (a) Overview of the detection platform. A U-shaped microfluidic DVD serves as detection platform. The hardware is derived from a standard DVD drive and controlled by a personal computer. The zoom-in area shows the differences between the testing zone and a blank. (b) Image of polymer beads of various sizes captured by DVD-LSM. (c) Working principle of the DVD-LSM. An additional photodetector is used to acquire the light transmitted through the disc in this system whereas the standard DVD drive only has a single photodetector to collect the light reflected from the disc. Adapted with permission from reference 41. Copyright © 2013, Royal Society of Chemistry.**

The two devices developed by Lange *et al.* and Ramachandraiah *et al.* are both capable of detecting and distinguishing particles as small as 1 μm in diameter.[40,41] Such microscopic imaging capability leads to the potential of constructing real-time healthcare examining system. It can be especially useful in situations where cellular- or molecular-level evaluation in blood samples or tissue biopsies are required. Beyond the comparable imaging resolution with standard light microscopes, these DVD-based systems can automate the preparation and handling of samples based on the spinning disk platform (combination of lab-on-a-disk systems).[40,41]

The researches described above emphasize and illustrate different approaches to the application of elegantly modified optical drives as optical detectors and microscopes. To operate these specially modified optical drives, customized programs have been developed to control the scanning area and even the scanning speed in order to automate these novel analytical platforms to be more user-friendly.

1.3.2. Derivatized optical discs for microsensor fabrication

In addition to the modification of optical drives for the preparation of various analytical devices, the “derivatization” of optical discs for the fabrication of nano- and microstructure materials and other analytical chemistry applications have been explored.[2] For example, in 2000, Angnes and co-workers pioneered the preparation of gold electrodes (so called “CDtrodes”) from gold CDs.[42] The reflective layers from gold CD-Rs are suitable as electrodes in electroanalytical chemistry. As the gold layer in a CD is sandwiched between a protective layer and a polycarbonate layer, so the disc is first cut with scissors into slices that are treated with concentrated nitric acid for a few minutes to fully expose the gold layer. They have shown by cyclic voltammetry that the electrochemical performance of the CDtrodes is comparable to that of commercial gold disk electrodes. They determined the concentration of mercury and copper in ocean or/and tap water by potentiometric stripping analysis, which utilizes a thin CDtrode as working electrode.[43] The main advantage of a CDtrode as compared to a commercially available electrode is that the former is simple to fabricate and cost-effective, which is applicable for disposable cells. Commercial electrodes (e.g., gold coated glass slides)

typically require more strict cleaning using corrosive acids (e.g., piranha solution) before use.

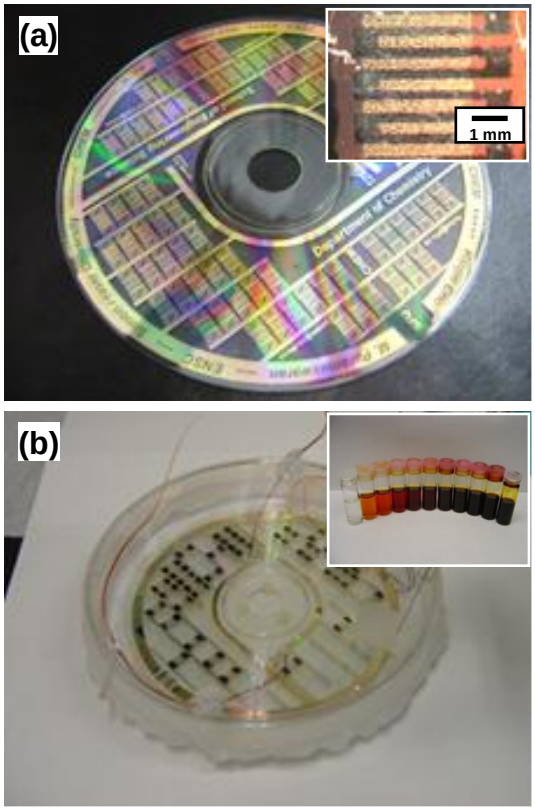

Figure 1.14. (a) Optical images of multiplex electrical sensors (for the detection of iodine vapor) constructed on a gold CD-R. The inset shows a patch of phospholipids (yellow) inkjet-printed on one set of the sensing electrodes. (b) Sealed chamber (Petri dish) enclosing the sensors into which the iodine solution is injected. The black dots on the CD are the conducting polymer films deposited at both ends of the microelectrodes for making the electrical connections. The inset shows iodine solutions at 11 different concentrations.[46] Reprinted with permission from reference 46. Copyright © 2007, Elsevier.

At the same time, our group has prepared high-quality self-assembled monolayers (SAMs) on gold CD-Rs[44,45] and found that old CDs can also serve as economical and valuable resources to fabricate electrodes for electrochemical applications. We further demonstrated a simple microfabrication technique for patterning microelectrode arrays with an inkjet printer. Before a gold CD-R is loaded on the tray for printing, its protective layer must be removed by immersing the entire disc in concentrated nitric acid. To create patterns on the gold films, we first printed an etching-resist layer (alkanethiol solution) with

the surface protected by SAMs, followed by a wet chemical etching protocol to remove the remaining unprotected gold layers. The disc is then exposed to 254 nm UV irradiation for 60 min to remove the remaining alkanethiolate SAM. After removing the etching-resist layer, an office inkjet printer deposits a well distributed phospholipid film on the electrodes to construct a prototype iodine vapor sensor (Figure 1.14).[46] As iodine is absorbed from the vapor phase into the phospholipid film, the conductivity of the film increases due to incorporation of I_2 or I_3^- into the lipid bilayer.

Independently, Daniel *et al.* have proposed a conceptually different patterning method to fabricate gold electrodes for microscale production.[47] Instead of printing SAMs on gold films as an etching-resist layer, they constructed electrodes from the gold CD-Rs using heat transferring masks and wet etching techniques.[47] In this approach, LaserJet toner layer served as a positive mask for the electrode. They first put waxed paper directly on the gold layer; LaserJet toner layer with the desired pattern can then be transferred from the wax paper onto the gold surface via thermal pressing. The toner layer prevents aqueous etching and protects the gold underneath. After the etching process, the toner layer is washed away with acetonitrile. This technique was used to develop a disposable microfluidic sensor consisting of two coplanar gold electrodes that are sandwiched between two polycarbonate slices to build an electrochemical cell. In addition, these authors described a concept in which CD-derived electrodes can be treated with microfluidic technology and further developed into a microchip-based electroanalytical system. The beneficial gains due to microfluidic technology include reduction of sample volume, control of mass transport, improved throughput and partial automation of assay analysis.

In 2012, Rusling and co-workers used the same fabrication technique (thermo-printing method) to develop the first ever microchip-based immunosensor for the detection of cancer markers .[48] In their work, they first printed the desired array pattern on a wax paper, and the toner layer is then transferred from the wax paper onto the gold during the heat transfer process. After etching away the unwanted gold layer, a second toner layer was heat-transferred again from wax paper to create hydrophobic microwells around the electrodes (Figure 1.15a). These microwells restrict the volume of liquid solution to ~1 µL for each of the sensors. Figure 1.15c shows the schematic view of the construction of the

entire device, i.e., a PDMS channel is embedded on top of the eight gold microelectrodes and then sandwiched between two poly(methylmethacrylate) (PMMA) plates. The limitation of the above mentioned fabrication techniques is their dependence on the printer resolution and the size of the toner particles.[46–48]

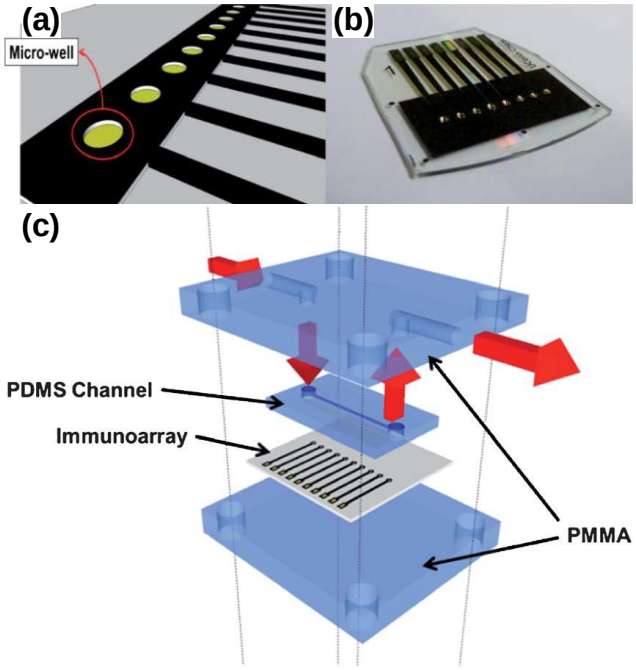

Figure 1.15. Microfluidic immunosensor for the electrochemical detection of cancer biomarker of interleukin-6. (a) Zoom-in computer-designed image of gold electrodes. The microwells are responsible for handling 1-µL droplets. (b) Full optical image of the microelectrodes fabricated from gold CD-Rs. (c) Schematic diagram of the construction of the microchip-based immunosensor. An immunoarray of eight gold electrodes (as shown in b) is sandwiched between two PMMA plates and one PDMS chip. The red arrows indicate the direction of fluid flow. Adapted with permission from reference 48. Copyright © 2011, Royal Society of Chemistry.

Recently, Kirkpatrick *et al.* described another patterning method on the gold film from the CDs that is assisted by photolithography. They spin-coated a positive photoresist on top of the gold-reflective layer to act as an etching resist material and subsequently removed unprotected gold with aqua regia.[49] The CD-based patterned electrodes were then integrated with the microfluidic network created by PDMS and used for amperometric detection.

Gold-reflective layers from CDs can be employed not only in electrochemical applications, but also in spectroscopic sensing. As the pre-grooved gold reflective layer in the commercial optical disc is sufficient to excite surface plasmons, Hiller and co-workers took advantage of this to develop a diffraction-grating-based surface plasmon resonance (SPR) sensor using the pre-grooved gold reflective layer in CDs.[50] Preparation of the grating platform involves a wet chemical treatment to remove the protective and lacquer layers on top of the reflective layer. The quantitation of analyte or observation of binding events with the use of a grating-based SPR sensor depends on the intensity of reflected light acquired at a selected angle of incidence and a single wavelength when the grating is placed perpendicular to the light path. While reading an SPR image, a brighter testing spot represents a higher concentration of the analyte while a dim one represents the opposite. Hiller *et al.* demonstrated that optical disc can be an alternative to thin films being used in conventional grating-based SPR sensors that often have expensive fabrication costs.

In other work, several research groups found that optical discs can be employed as alternative surface-enhanced Raman spectroscopy (SERS) substrates.[51–53] The recent commercially available SERS substrate (Klarite™) has gold-coated nanostructured silicon on its active site. SERS signal enhancement arises from “hotspots” located between nanostructures. Similarly, the periodic metal grating patterns on the optical disc surface can enable coupling of surface plasmons with the laser excitation to provide intense localized electromagnetic radiation and therefore enhance Raman signals. To fabricate the SERS-active substrate, Song *et al.* first used concentrated HCl to reveal the reflective silver layer in a CD.[52] The silver layer was further roughened by electrochemical oxidation-reduction cycling to improve SERS activity from the formation of silver particles on the CD surface. The approach of Nieuwoudt *et al.* is different, i.e., they physically cut a BD-R into 1-cm^2 sections, peeled off its protective layer carefully to obtain the grating surface, and then sputtered a gold thin film on top.[53] As noted in section 1.2.1, BDs have the highest nanostructure density which improves SERS signal enhancement. They found a 660-fold increase in this enhancement on melamine detection on the gold-sputtered BD-R which is comparable to the commercially available Klarite™ SERS substrate (790-fold increase). Klarite™ is expensive for large-scale routine analysis in industry, whereas disc-modified SERS substrate is rather inexpensive and easy to prepare.

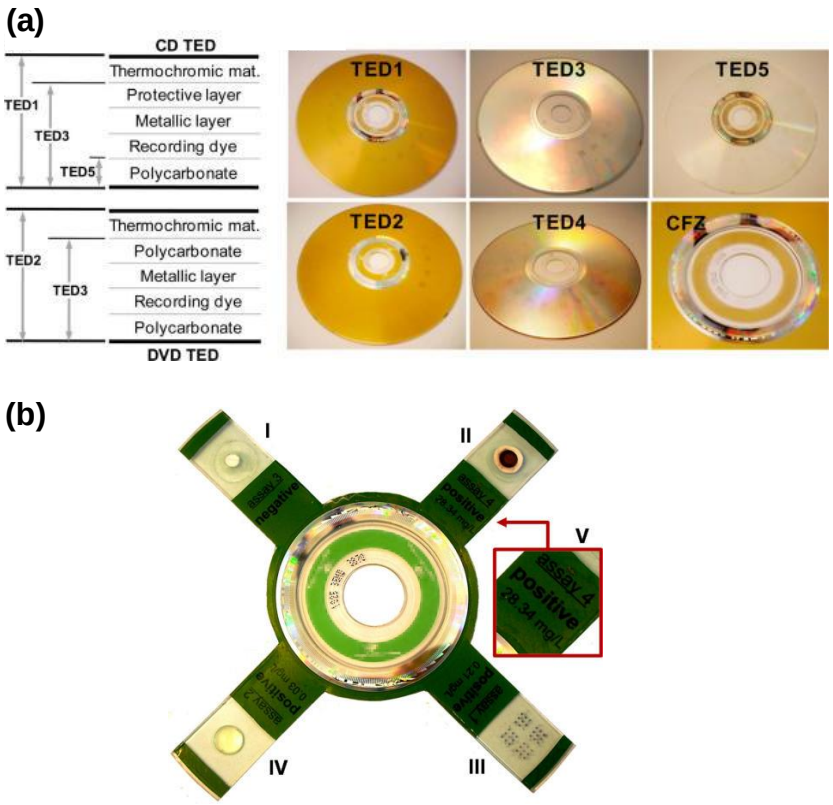

Figure 1.16. Thermochromic etching disc (TED)-based analytical platform. (a) Layered structures of TED. The control feature zone (CFZ) is located at the inner part of the disc which provides coordination information for laser pickup head movement. (b) Four different analytical systems on TED: (I) turbidimetry, (II) immunofiltration, (III) microarray fabrication, and (IV) cell culture preparation. The analyzed results can be printed on the photochromic coating of the same disc (V). Adapted with permission from reference 54. Copyright © 2014, American Chemical Society.

Aside from creating nanostructures on an optical disc for spectroscopic sensing, Avella-Oliver *et al.* proposed a total analysis system with a thermochromic etching disc (TED) known as LightScribe. This system allows for quantitative detection based on attenuated analog signal reading using a customized disc drive to acquire analog signals directly from the photodiode (Figure 1.16).[54] In contrast to normal optical discs, the LightScribe has a thermochromic coating on the label side which darkens when irradiated with a 780 nm laser. Another special feature of the LightScribe system is that the scanning process is track-independent which allows to control movement of the laser pickup head precisely. Specifically, the position of the disc can be recognized by continuously

irradiating the control feature zone, a circular coordinate code located in the inner circle of the disc. Fabrication of each layer of the disc allows the use of different materials as sensing substrates. Avella-Oliver *et al.* demonstrated that the photochromic layer can be dissolved in an ethanol bath in 10 minutes, and a polycarbonate disc can be prepared from LightScribe by physically peeling off both the photochromic and metallic layers. After the disc preparation, the polycarbonate layer is adapted to function as a bioanalytical platform for immunofiltration, microassay/cell culture preparation, and turbidimetry. Cell counting in the turbidimetry chamber can be performed by scanning the disc and measuring a loss of transmitted light intensity due to the scattering effect of particles in the chamber.

The examples discussed in this section demonstrate that optical discs can provide versatile substrate materials for both for electrochemical and spectroscopic studies (e.g., surface plasmon resonance and surface enhanced Raman spectroscopy). It has been found that the performance of biosensors adapted from ordinary optical discs is comparable to that of platforms available commercially. Other special optical discs can be "converted" into multiplex detection platforms for biomedical applications.

1.3.3. *Bona fide* disc and drive for molecular sensing

In general, adaptation of an optical disc can easily make it incompatible with a conventional optical drive, destroying the intrinsic functions of data storage and retrieval. As a result, the main challenge in the development of a *bona fide* optical disc-based analytical platform is that the variation of surface morphology on a disc needs to be minimized to avoid adverse effects in its optical and mechanical properties so that the disc can still be recognized by an ordinary optical drive. As such, the most innovative approach would be a software approach that make use of *bona fide* optical discs and standard computer drives.

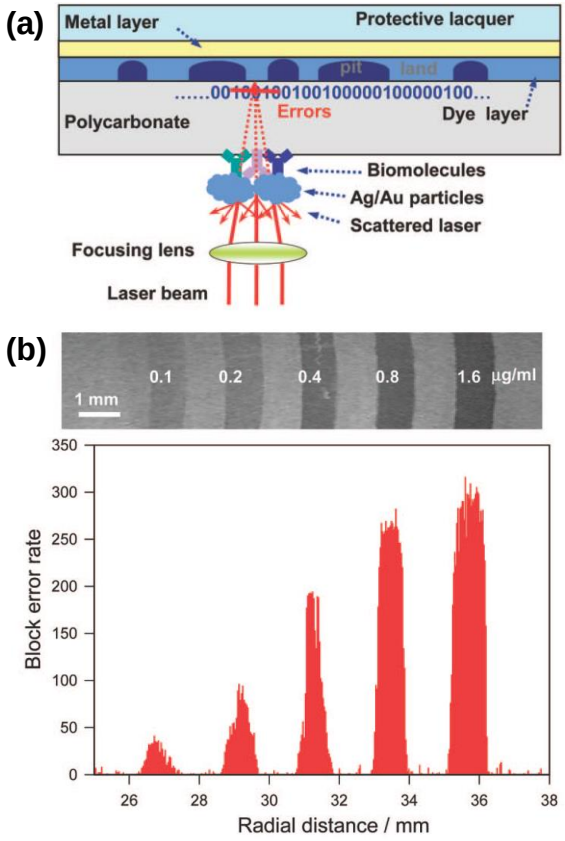

Figure 1.17. (a) Digital reading of disc-based bioassays with a standard computer drive: the biomolecule/nanoparticle conjugates block the reading laser and generate significant errors. (b) Quantitation of biotin/streptavidin binding assays on a CD-R; the distribution of block error rate on a disc modified with five strips of biotin/streptavidin binding lines. Adapted with permission from reference 55. Copyright © 2008, American Chemical Society.

In 2008, our group developed first CD-based digital readout protocol for quantification of biomolecules without modification of either the hardware (neither the disc nor the drive) or the software.[55] The working principle and applications of computer-readable disc-based bioassays were reviewed in 2013.[2] Extended applications in environmental and biomedical fields have been published recently.[56,57] In brief, any object with a size greater than one quarter of the laser wavelength (1/4 λ) on the disc surface can lead to significant laser disruptions, and the computer will recognize disruptions as reading errors.[2] Inspired by the delicate data protection and retrieval mechanism inherent in optical disc systems, we converted these disruptions into quantitative information. Biomolecules are first constructed on an optical disc and, since most of them are too small

to cause laser scattering, assay signals are amplified by a customized silver-staining protocol, such that the amount of analytes in the sample is directly proportional to the number of reading errors being created on the disc. Several disc quality diagnostic software programs can be downloaded online free of charge, including IsoBuster, PlextorTool, and PlexUTILITIES, which all have the ability to depict the propensity of reading errors. Since the playtime of an audio file and the logical position of error signals are correlated with the specific physical location of the binding sites on a disc,[55,58,59] we can identify where binding events have occurred, i.e., if there is any laser disruption.

With this digital readout system, we initially tested the streptavidin–biotin binding, DNA hybridization, anti-human IgG/human IgG binding assays. A ppb-level lead detection method with pre-assembled DNAzymes was subsequently reported.[2,60] A schematic diagram of the digital reading of a bioassay on a CD is shown in Figure 1.17a. The silver particles on the surface interrupt the laser reading and generate reading errors. Figure 1.17b shows a quantitative biotin-streptavidin binding assay on a CD-R, clearly the reading errors rise monotonically when the target concentration is increased.

Imaad *et al*. made significant progress on the construction of digital microfluidic CDs for counting microparticles and living cells (Figure 1.18).[61] To build a microfluidic network without producing a significant quantity of reading errors, and thereby allow reading and recognition by a conventional optical drive, the PDMS microfluidic layer within the disc needs to maintain a uniform thickness (total maximum thickness of 1.2 mm). As Figure 1.18a shows, when particles or stained cells are introduced into the microfluidic channel (in position b), they cause interference of converging laser beams which are recognized as reading errors in the encoded digital data. In contrast, the error rate is lower in the absence of particles or stained cells (Figure 1.18b). The number of reading errors obtained with an open-source disc quality check software (QpxTool), is proportional to the number of particles or cells present in the microfluidic channels.

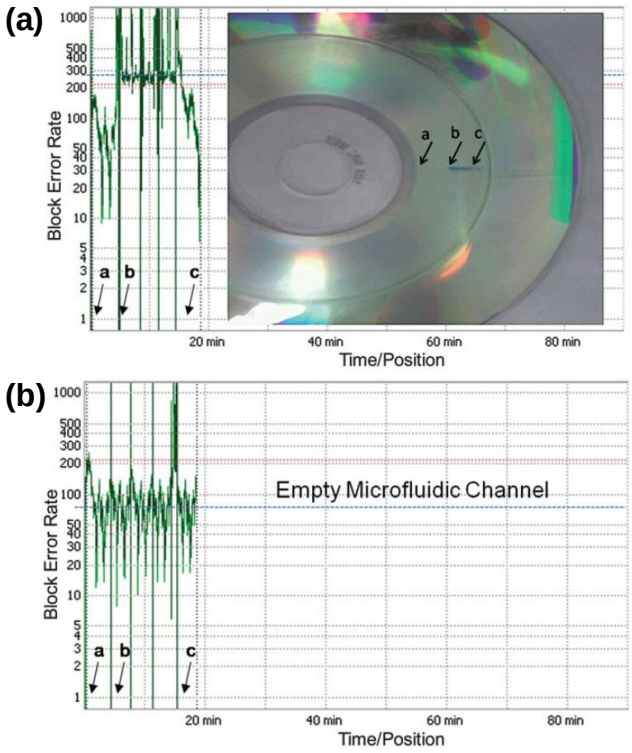

Figure 1.18. Detections of immobilized microparticles in the microfluidic CD. (a) Photograph of a microfluidic CD after blue microparticle loading and the corresponding data block error rate picked from the microfluidic CD with the channel partially loaded with particles. Three regions are marked by the arrows including 'a'—the start position of digital data on CD; 'b'—the start position of the region in the microfluidic channel with microparticles; and 'c'—the start position of the region in the microfluidic channel without particles. (b) Data block error rate picked up from the microfluidic CD with an empty microfluidic channel which is only exposed to a PBS buffer solution without microparticles. Reprinted with permission from reference 61. Copyright © 2011, Royal Society of Chemistry.

Recently, Ivanov *et al*. developed the application of their CD-based optico-acoustical (music derived from reading errors) sensing platform for recognizing various types of proteins such as bovine serum albumin, heme-containing myoglobin, monoclonal antibody against viral protein marker of hepatitis B, and membrane-bound cytochrome P450scc.[62] Based on the similar digital reading error principle introduced above (Yu *et al.*[2] and Imaad *et al*.[61]), Ivanov and co-workers found that binary errors provide an ideal metric to connect molecular recognition assays to computational systems. The signal readout strategy for this biosensor is the transformation of digital error signals to musical acoustics.

Customized software was developed (in C language) for measuring the noise level and constructing error distribution diagrams. To transform these error messages from each data sector into acoustic form, a MIDI-interface software was used for acoustic reproduction and melody storage whereby each acoustic signal with particular frequency was assigned values from 0 to 127 (the higher the value, the higher the frequency of a sound). By looking at the error distribution diagram or listening to the melody in the audio file, one can identify and quantify the numerous biomolecular complexes. In their work, they demonstrated that various protein-binding events can be determined, or distinguished, based on a propagation of erroneous bits within a string of bits arising from the changes of optical density on the disc surface. As the concentration of the protein increases, the number of errors being registered per sector in the CD also increases proportionally and appears in the form of higher-frequency acoustic signals. However, it should be noted that an optico-acoustical biosensor is insufficient to register proteins without amplification by silver staining. The reliability of this method has been confirmed by mass spectrometry. This optico-acoustical detection platform illustrates interesting musical data presentation, different from the traditional graphic presentation. Normally, people analyze data optically, yet auditing response can now also identify the results.

The software approach is a breakthrough in advancing optical disc technology-based analytical devices for on-site chemical analysis and POC medical diagnosis. It provides advantages such as no modifications to either the optical drive or operation software are needed, which leads to the accessibility to every user to read biomedical assays with an ordinary computer drive. Moreover, the surface chemistry involved in preparation of these disc assays is simple and mild that can be carried out by non-specialists. Based on the advantages mentioned above, this kind of analytical platforms would particularly benefit people who live in a resource-limited and/or rural areas.

1.4. Objectives of this thesis

As emphasized above, point-of-care diagnostic tools will greatly benefit people who live in resource-limited and rural areas, as they can perform self-monitoring tests and examinations at home or in a doctor’s office, with test results available immediately. Consumer electronics (e.g., office scanners, smartphones, disc players) are promising

candidates to be “converted” into cost-effective and portable POC devices. From 2008 to 2013, our group has been working on the development of molecular sensing platforms based on *bona fide* CDs and computer drives. As described in Section 1.2.1, DVD and BD systems utilize shorter-wavelength lasers and larger numerical aperture objective lenses, which offer better resolution than the CD setup. The objective of this thesis is not only to examine the performance of DVD- and BD-based diagnostic systems (not yet tested previously) compared to traditional analytical methods (colorimetric determination and ELISA tests), but also to explore their potential applications for medical diagnosis (real sample tests). In addition, since there is a special hard coating material on top of a BD-R surface, a new surface activation protocol is required for probe immobilization.

This chapter is an introductory chapter and followed by this; the next chapter of this thesis, DVD signal readout mechanism as well as the performance of DVD technology-based quantitative pregnancy tests are described (Chapter 2). In Chapter 3, the characterization of a BD surface and its surface activation protocol will be first discussed. Performance of the BD technology-based multiplex cardiac marker diagnostic platform will be described with discussion of its improved reading principles (Chapter 3). Chapter 4 concludes this research and suggest future directions in advancing optical disc technology-based analytical devices for on-site chemical analysis and POC medical diagnosis.

Chapter 2.

DVD technology-based molecular diagnostic platform: quantitative pregnancy test on a disc*

2.1. Introduction

As mentioned in Chapter 1, several research groups started to explore molecular detection applications of DVD technology during the last decade. For example, Maquieira and co-workers fabricated multiplexed DVD microimmunoassays. They used an external amplification / detection board in conjunction with a DVD drive circuit to acquire analog signals directly from the photodiode.[63] They also developed microsensor arrays to detect biotoxins (microcystins) in river water, and they made a DNA microarray to simultaneously detect traces of multiple food allergens with the same method of signal readout.[64,65] Gopinath *et al.* reported a conceptually different bioDVD platform that utilizes the optical interference of multilayer nanostructures sputtered on a DVD surface (read by a special reflectance-based optical reader).[19,20] Ramachandraiah *et al.* developed a new HIV diagnostics device by converting a commercial DVD drive into a laser-scanning microscope to identify and count the number of CD4+ cells in blood samples.[41]

Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein hormone produced during the initial stages and throughout the main period of pregnancy; the qualitative hCG strip test is today's gold standard for detecting pregnancy.[66,67] Quantitative hCG tests can be used to monitor abnormal pregnancy, gestational trophoblastic diseases, and testicular cancer.[68,69] However, the current quantitative hCG test is mainly based on serum immunoassays, which have to be carried out in a laboratory or hospital

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*Reproduced in part with permission from X. Li, S. Weng, B. Ge, Z. Yao and H.-Z. Yu, DVD technology-based molecular diagnosis platform: quantitative pregnancy test on a disc, *Lab Chip*, 2014, **14**, 1686–1694.[59] Copyright © 2014 Royal Society of Chemistry. This project was initialized by X. Li, who started the experiments, which were finished by S. Weng. B. Ge and Z. Yao provided technical support for the ELISA experiments. X. Li, S. Weng and H.-Z. Yu wrote the manuscript.

and require trained medical professionals and special instruments. Processed results are typically delivered in one to two days.[70] In this chapter, the development of a bioassay platform based entirely on standard DVDs and conventional computer drives for quantitative molecular diagnosis is described. We used hCG detection in standard physiological buffers and in urine samples as a trial system.

2.2. Experimental section

2.2.1. Materials and reagents

The hCG standard samples (100 mIU/mL), anti-hCGα monoclonal antibody (Mab), anti-hCGβ Mab, horseradish peroxidase (HRP)-conjugated anti-hCGβ Mab, FSH (follicle-stimulating hormone), and TSH (thyroid-stimulating hormone) standard samples were provided by Biogate Laboratories Ltd. (Burnaby, Canada). Urine samples from healthy pregnant women (28 to 38 years old) were obtained from the No. 3 Hospital, Shanxi Medical University (Taiyuan, China). Samples were stored in aliquots at -20 °C to insure the hCG hormones are not degraded. The biotin labeling kit-NH_2 was purchased from DOJINDO Molecular Technologies, Inc (Rockville, MD). The Sylgard 184 Silicone Elastomer kit was purchased from Dow Corning Corporation (Midland, MI). *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-morpholinoethane sulfonic acid (MES), HRP and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. MPEG3-NH_2 ($C_7H_{17}NO_3$) was purchased from ChemPep Inc. (Wellington, FL). 3,3',5,5'-tetramethybenzidine (TMB) solution was provided by Moss Inc. (Pasadena, MD). Deionized water (>18.3 MΩ cm) was produced with a Barnstead Easy Pure UV/UF compact water system (Dubuque, IA).

The buffer solutions had the following compositions. Activation buffer: 0.1 M MES, and 0.9% NaCl, pH 4.7; immobilization buffer: 100 mM phosphate buffer, 150 mM NaCl, 5% glycerol, and 2 mM NaN_3, pH 7.4; blocking buffer: 100 mM phosphate buffer, 150 mM NaCl, 4% BSA, and 2 mM NaN_3, pH 7.4; washing buffer: 100 mM phosphate buffer, 500 mM NaCl, 0.8% BSA, 0.1% gelatin, 0.05% tween-20, and 2 mM NaN_3, pH 7.4.

Human body fluids (urine samples) were handled as bio-hazardous according to the University Biosafety Program Manual. Operator needs to wear appropriate personal protective equipment, such as gloves, lab coat, and protective eyewear. The waste (pipette tips and Eppendorf tubes) was treated as potentially bio-hazardous and placed in clear autoclave bags, discarded after autoclaving.

2.2.2. Surface activation and assay preparation

Before the surface activation step, video data was burned on a blank DVD-R (MAM-A DVD-R 8 x Gold) and then cleaned by rinsing with ethanol and deionized water. Since the DVD substrate is made of polycarbonate, the surface was activated by UV (185/254 nm) irradiation in UV/ozone cleaner (PSD-UV, Novascan Technologies, Inc.) for 15 min and was kept in the chamber for 20 min. A PDMS plate with embedded microfluidic channels (500 μm × 50 μm × 15 mm) was placed on the DVD surface (Figure 2.1a). 1 μL of 100 mM EDC and 25 mM NHS in the activation buffer were injected into each channel and incubated at room temperature for 3 h. The solution in the microfluidic channels was removed via vacuum suction, and the surface was washed with deionized water. As the PC surface became hydrophilic upon UV/ozone activation, the physical attachment of the PDMS plate on DVD is robust. Extreme care should be taken when adding solutions to the microchannel inlets, particularly to make sure that the pipets are perpendicular to the disc surface.

To prepare the hCG sandwich assay (Figure 2.1b), anti-hCGα Mab (50 μg/mL, 1 μL/channel) in the immobilization buffer was injected into the channels and kept for 1 h in order to immobilize the capture antibodies onto the DVD surface. Unbound anti-hCGα Mab was removed by vacuum suction followed by washing with deionized water. The unreacted carboxyl groups were blocked by reaction with 1.0 mM MPEG3-NH_2 in the washing buffer (1 μL/channel) for 1 h. After that, the hCG samples in the immobilization buffer were injected into the channels (1 μL/channel) and incubated for 1 h. Biotin-labeled anti-hCGβ Mab (0.05 μg/mL) in the washing buffer was then added into the channels (1 μL/channel) and kept for 1 h. The DVD was then rinsed with the washing buffer twice, followed by a signal amplification step.

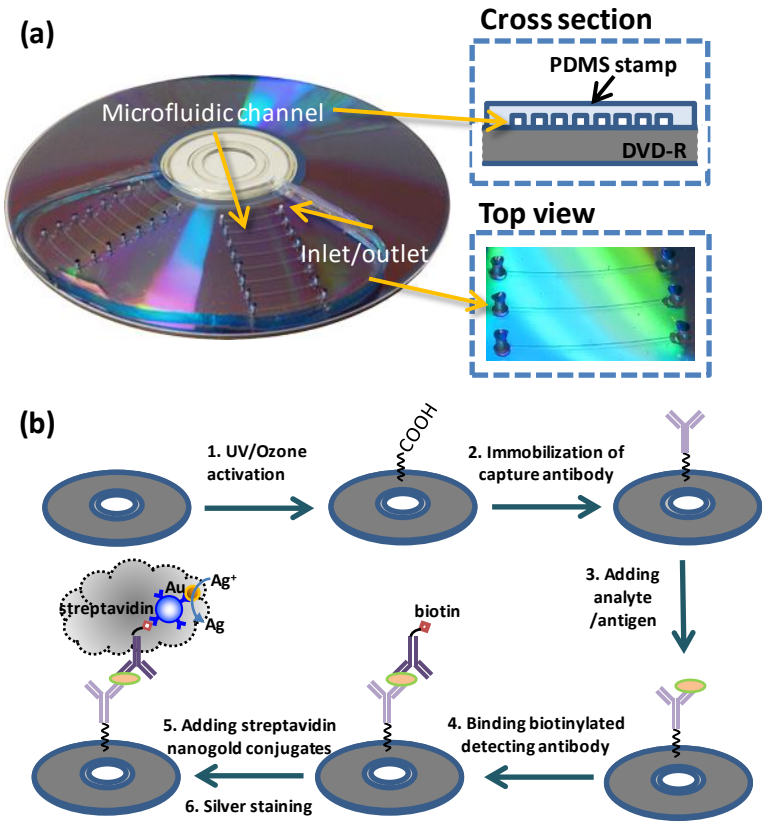

Figure 2.1. **(a) Microfluidic approach to prepare line arrays of DVDassays. PDMS plates with embedded microchannels were used to deliver the reactants and washing buffers. The left image shows a PDMS plate attached to a DVD surface and the right pictures show the cross section (schematic) and top view. (b) Surface reactions on a DVD to prepare computer-readable, sandwich-format immunoassays: 1. UV/ozone activation to generate carboxyl groups on a DVD-R; 2. Immobilization of capture antibodies via amide coupling; 3. Binding analytes (antigens) on the surface bound antibodies; 4. Binding biotinylated detection antibodies; 5. Binding nanogold streptavidin conjugates; 6. Silver staining for signal enhancement (reduction of Ag^+ to metallic Ag). Reproduced with permission from reference 59. Copyright © 2014, Royal Society of Chemistry.**

2.2.3. Signal amplification and signal readout

Nanogold-streptavidin conjugates (0.8 µg/mL) in washing buffer were injected into the microfluidic channels (1 µL/channel) and kept for 1 h at room temperature. The DVD was then rinsed sequentially with washing buffer (5 min) and deionized water (2 × 5 min). The PDMS chip was then removed from the DVD surface, followed by silver enhancement

reactions on the surface with a mixture of equal volumes of 0.4% (m/v) silver nitrate solution and 1% (m/v) hydroquinone (dissolved in 10 mM citrate acid buffer at pH 5.35). The signal-enhanced DVD was washed with deionized water and dried under nitrogen gas.

The bioassay DVDs were read with a standard optical drive (PLEXTOR PX-LB950UE external Blu-ray) and a free disc-quality diagnostic program (PlexUTILITIES 1.3.3.1, http://www.goplextor.com/) was used to check for error distributions of both PIE and PIF using the BLER mode. This program controls the optical drive to run at 16 x speed, such that it typically takes 5 min to screen the entire DVD. The data was exported to Microsoft Excel or Sigma Plot for further analysis (e.g., to integrate the PIF counts of each binding band and determine the corresponding PIF density).

2.2.4. ODR determination and ELISA validation

The DVD bioassays were scanned with a reflective scanner (Epson Perfection 1250). Adobe Photoshop CS4 software was used to analyze the optical darkness ratio (ODR) of the strips on the DVD. This was carried out using a histogram tool to determine the average luminosities of the assay site and the background, respectively.

A 96-well ELISA plate (microtiter plates, Shenzhen Jinchanhua Co.,Ltd.) was first coated with capture antibody (100 µL/well of 2 µg/mL anti-hCGα Mab in 0.05 M carbonate buffer, pH 9.6) overnight at 4 °C. After coating, the plate surface was blocked by 2% BSA (dissolved in 0.1 M phosphate buffer, pH 7.4, 200 µL/well) for 2 h at room temperature and then washed with the washing buffer (200 µL/well) five times. Urine samples (100 µL/well) were then introduced and the plate was incubated for 1 h at 37°C. After washing, the HRP-conjugated anti-hCGβ Mab (0.5 µg/mL, 100 µL/well) was added into each well followed by incubation for 1 h at 37 °C. After repetition of the same washing step, TMB solution (2.5 mM, 100 µL/well) was introduced to the wells and kept for 20 min before adding the stop solution (2 M H_2SO_4, 50 µL/well). The absorbance change at 450 nm was then determined with a ChroMate Microplate Reader (Awareness Technology Inc., Palm City, FL).

2.3. Results and discussion

2.3.1. Surface characterization

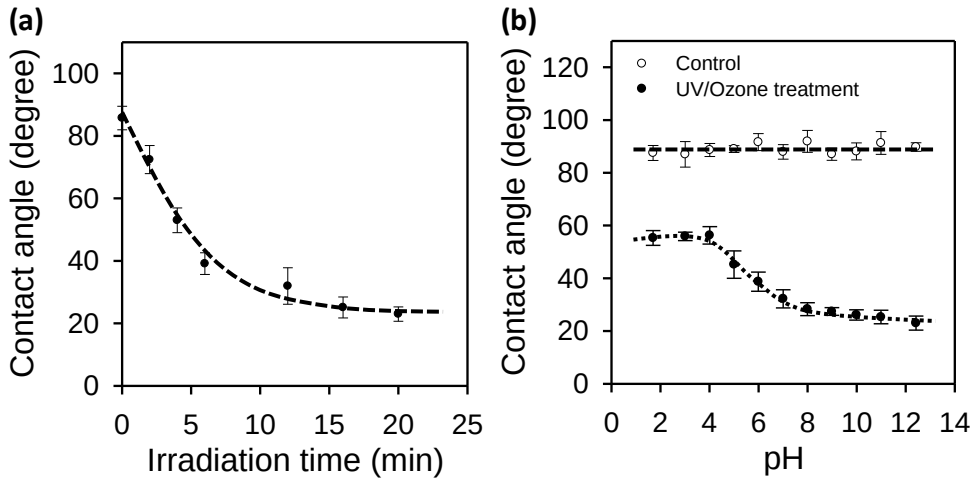

Figure 2.2. (a) Dependence of water contact angle on a DVD-R surface as a function of the UV/Ozone activation time; (b) Contact angle titration of an activated DVD-R surface (15 min UV/ozone treatment) and non-activated surface. This confirms the formation of reactive COOH groups besides other polar functionalities (e.g., -OH). The dashed and dotted lines are to guide the eyes only. The error bars shown in the plots are standard deviations from three repeated measurements on the same DVD.

The main substrate of both CDs and DVDs is polycarbonate, so UV/ozone activation method previously developed in our group was applied to activate the surface. To determine the optimum irradiation time, the dependence of the water contact angle on a DVD-R was plotted as a function of the activation time. As shown in Figure 2.2a, the water contact angle dropped from 86 ± 4° to 25 ± 3° within 15 min of UV irradiation (with ozone). The surface became more hydrophilic when more polar groups such as hydroxyl (OH) and carboxyl (COOH) are introduced. In this case, we used contact angle titration to confirm the formation of surface reactive carboxyl groups after surface activation. Figure 2.2b shows that the contact angle remains constant (89 ± 3°) on untreated surface throughout titration from pH 2 to 12. In comparison, the contact angle titration on the activated DVD surface is a smooth transition (55 ± 2° to 26 ±2°) from pH 4 to 9. The change in the hydrophobicity of the surface reflects ionization of surface carboxyl groups.

2.3.2. hCG-DVD assay design

After studying DVD surface activation, an hCG sandwich-format immunoassay was designed on the surface of a regular DVD for signal reading with a standard optical drive (Figure 2.3). The immobilization and binding of antibodies/antigens and the microfluidic approach (using a PDMS stamp with embedded microchannels) to prepare line arrays of binding strips on a standard DVD-R followed established procedures as described in the experimental section (Figure 2.1b).[71] In particular, two different monoclonal antibodies were employed as recognition elements: capture antibodies to specifically recognize hCG-alpha subunits (hCGα) and detect antibodies for the recognition of beta subunits (hCGβ).[72] To prepare a hCG sandwich assay, the polycarbonate surface of a regular DVD-R was first activated via UV (185/254 nm) irradiation, followed by treatment with EDC and NHS. After the activation, anti-hCGα monoclonal antibodies were immobilized on the disc surface via an amide-coupling reaction. During the sample testing, surface-immobilized anti-hCGα monoclonal antibodies should capture regular hCG and hyperglycosylated hCG, which would then be detected by the biotin-labelled anti-hCGβ monoclonal antibody. The streptavidin nanogold conjugate was bound to the surface via biotin-streptavidin interaction, and this was followed by silver staining treatment. This signal enhancement step is needed because biological macromolecules are too small (<<1/4 λ, λ = 650 nm for DVD) to induce significant disruption of the reading laser in standard optical drives. Using a the commercial Nanoprobes LI silver enhancement kit, it takes at least 30 min to obtain a readable signal,[16,73] which is too slow to be acceptable for hCG POC testing. In order to improve the detection efficiency, customized silver enhancement solutions (see experimental section 2.2.3) were developed by testing different concentrations of silver nitrate and hydroquinone (reducing agent). The prepared DVD assay was then tested digitally with free DVD-quality diagnostic software.

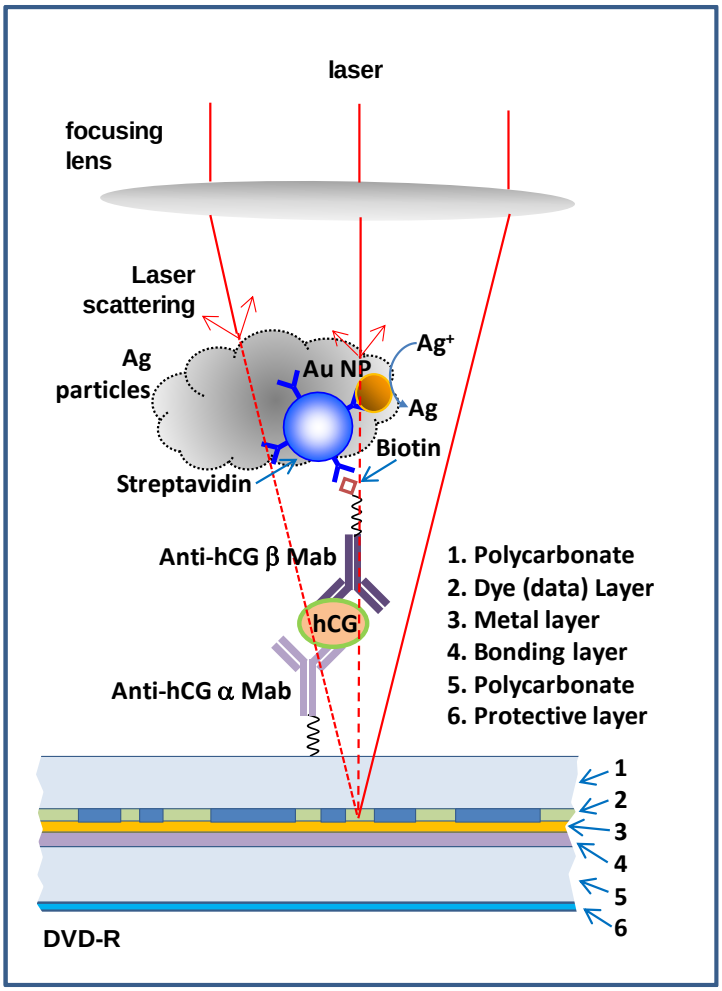

Figure 2.3. **DVDassay design and signal reading principle. The immunoassay to detect hCG was constructed on the polycarbonate surface of a standard DVD-R in a sandwich format; anti-hCG α Mab and anti-hCG β Mab were used as the capture and detecting antibodies, respectively. Gold nanoparticles (Au NP) conjugated with streptavidin were introduced to the assay through the interaction with biotin tags (on the detecting antibodies), promoting the reduction of Ag^+ to metallic silver particles. Thus deposited, rather large Ag particles (> 300 nm) would interrupt the laser reading process when played in a conventional optical drive, and would create digital reading errors (proportional to the antigen/analyte concentration). Reproduced with permission from reference 59. Copyright © 2014, Royal Society of Chemistry.**

2.3.3. Digital reading protocol

The previously developed error-reading protocol with pre-recorded audio files on CD-R presented problems when used for DVD-R[55], because of the much greater DVD storage capacity (4.7 GB vs. 700 MB). We therefore explored the option of error analysis with digital video files, which typically occupy 1 GB or more to keep the writing step feasible. Similar to audio files on a CD-R, a byte error occurs when one or more bits in a byte have a value that deviates from the originally recorded one. For commercial DVDs, this may occur during the recording process, or by scratches and smudges.

Based on the error detection principle discussed in the previous chapter, the detection mode of the DVD error reading protocol was evaluated by marking a DVD-R with a series of ink spots of different sizes (Figure 2.4). Figures 2.4b and 2.4c show the PIE/PIF test results of pre-recorded DVD-R. The data were collected using the BLER testing mode of PlexUTILITIES 1.3.3.1. The software plots the PIE/PIF counts for every 0.03125 megabyte (MB) (an ECC block), as the DVD drive reads each ECC block to determine the error counts. As shown in Figure 2.4b, all 12 peaks corresponding to the ink stains can be identified with no difficulties, but the background of PIE counts are very high (S/N > 3.0). In contrast, the PIF background noise of the recorded DVD is much lower than the PIE, and in most positions the PIF counts are zero (Figure 2.4c). The noise is almost negligible because more than 5 bytes are needed to generate a PIF signal. Therefore, counting PIF is more appropriate for the quantitation of any introduced reading errors. We also discovered that broader and higher peaks correspond to larger/darker ink spots. The smallest spot tested at the far left of the disc (~180 μm in diameter) still produced a distinct peak in the PIF reading, indicating the lateral resolution of a DVD error reading is substantially better than that of a CD (~250 μm).[55] However, this is not comparable to the lateral resolution reported by Ramachandraiah *et al.*, in which the reading laser of a modified DVD drive was directly focused on the surface of the disc to achieve the lateral resolution of 1 μm. In our system, the lateral resolution of the DVDassay is limited by the laser spot (the focal point located at the dye layer, Figure 2.3) on the polycarbonate surface.

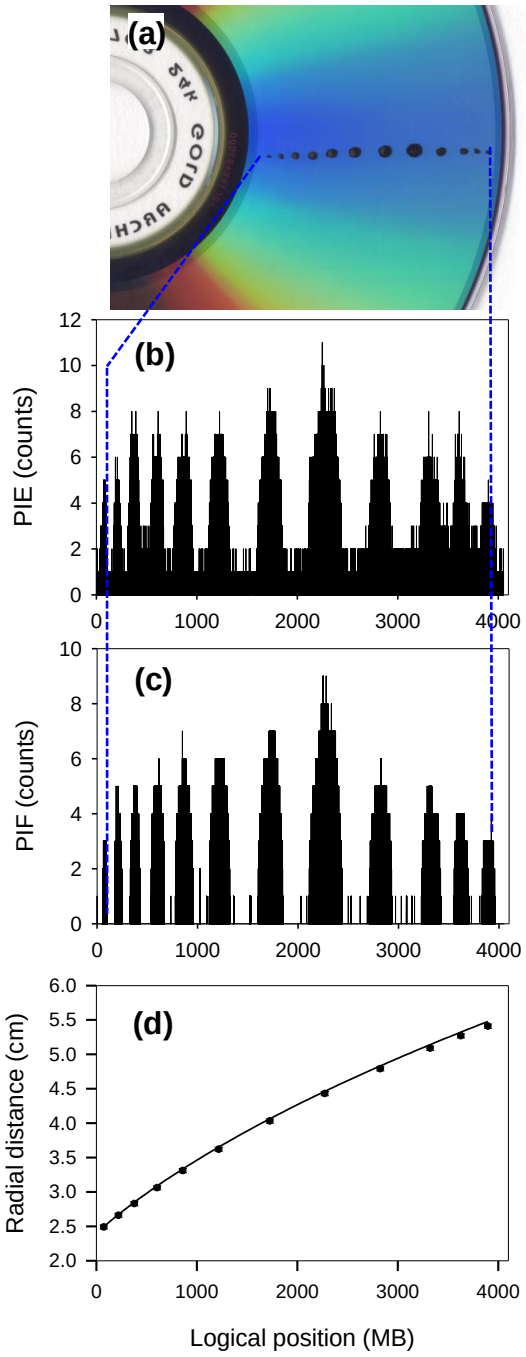

Figure 2.4. **(a) Optical image of a DVD-R with ink stains of different sizes at different locations; (b) PIE reading results of the "stained" DVD-R; (c) PIF reading results of the same DVD-R; (d) Correlation between the logical position and radial distance of the ink stains on the tested DVD-R. Reproduced with permission from reference 59. Copyright © 2014, Royal Society of Chemistry.**

The relationship between the radial distance (the distance from the spot location to the DVD center) and the logical position of the error peaks is described below. In Figure 2.4d, the two measured values are correlated by the equation

$$r = \sqrt{\frac{x}{4482} \times (5.8^2 - 2.4^2) + 2.4^2} \quad (2)$$

where *r* is the radial distance of DVD (cm) and *x* is the logical position (MiB). A typical DVD has a data zone with an outer radius of 5.8 cm and an inner radius of 2.4 cm.[1] This equation is derived based on the capacity of a standard DVD-R and its programmable area where the digital data is located. Since the location of programmable area is started from a radius of 2.4 cm, *r* has a value equal to or higher than 2.4. According to this equation, one can identify the physical locations of any binding sites on the DVD surface that cause a significant disruption of the laser. For the 4.7 GB DVD-R (with a capacity of 4482 MiB) used in this study, as example, the last error peak occurs at 3896 MB corresponding to the ink spot located 54.8 mm from the center of the disc. Being able to determine the physical location of binding sites is essential to the preparation of multiplex assays.

2.3.4. DVD-based bioassay for hCG quantitation

We first designed a “reproducibility” hCG test assay on an eight-channel PDMS plate to record the binding strips of 5.0 mIU/mL hCG each in odd-numbered channels, and for four blanks (buffer solution only) in even-numbered channels. In addition to the optical image of the assay (Figure 2.5a), the PIF reading plot (Figure 2.5b) and the integrated PIF counts per unit assay area (PIF density) (Figure 2.5c) are shown. The four quadruplicate hCG testing lanes (channels 1, 3, 5, and 7) show reproducible, distinct signals, in clear contrast to the background (channels 2, 4, 6, and 8). An hCG concentration of 5 mIU/mL was chosen for this experiment, since below this concentration most commercial pregnancy test strips show a negative result, i.e., no pregnancy. The average signal was 254±15 PIF counts/mm^2, which is more than 5 times higher than that of the blanks (57±20 PIF counts /mm^2).

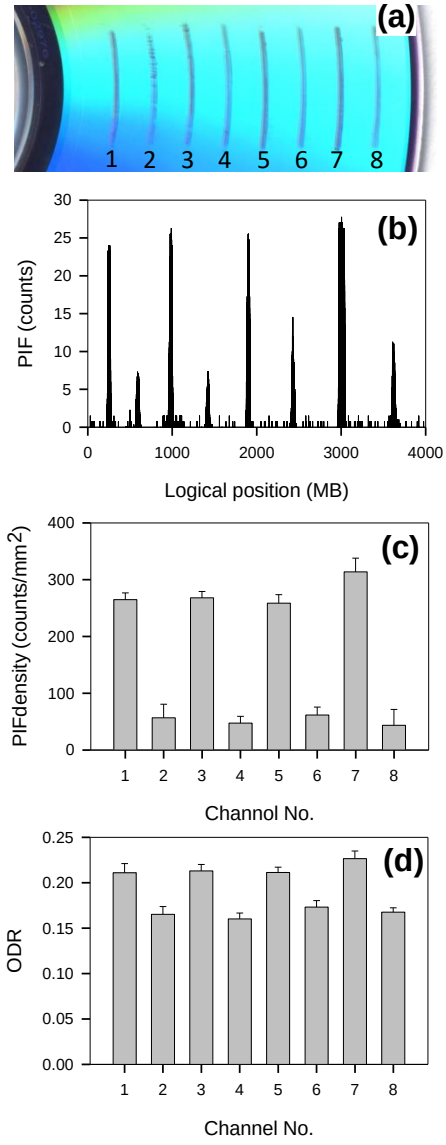

Figure 2.5. **(a) PIF reading of a hCG assay on DVD. Channels 1, 3, 5, and 7 are hCG standards (5.0 mIU/mL) and channels 2, 4, 6, and 8 are blanks (buffer only). (b) Corresponding PIF density of the hCG assay (integrated PIF counts normalized to the assay area). (c) ODR plot of the test results, based on the optical image shown on the inset of (a). The error bars shown in these plots are standard deviations determined from three measurements of the same DVDassays.**

For direct comparison with traditional assay methods, we also determined the ODR (optical darkness ratio) value of each binding strip (Figure 2.5a). As a colorimetric protocol, the ODR value is defined as

$$ODR = \frac{I_b - I_s}{I_b} \quad (3)$$

where I_b is the luminosity of the background and I_s is that of the binding site.[73] I_b and I_s have values ranging from 0 to 255, where a smaller value indicates a darker binding site (*vice versa*). As shown in Figure 2.5d, the ODR reading does not provide unequivocal distinction between the sample and the background signal. The ODR obtained from the sample (0.215 ± 0.007) was only 30% higher than that from the background (0.166 ± 0.005). On the other hand, digital error reading (Figure 2.5c) provides a reproducible result with clearer separation between sample and background signals. This indicates that digital readings are more significant and reliable than traditional colorimetric methods (ODR).

One of the goals of this study was to explore the feasibility of the DVD bioassay platform for quantitative determination of hCG concentrations in clinical samples (urine samples from pregnant women). A series of hCG standards, from 0.78 to 25 mIU/mL in physiological buffers were therefore tested to cover the typical range of hCG levels in early pregnancies[68,74,75] on the same DVD-R. Figure 2.6a shows the design for hCG assays. hCG standards (No.1-7) and normal urine samples (No.8) are shown on the left-hand side of the image, unknown urine samples (No. 1'-7') from different pregnant women on the right-hand side. The distribution of PIF counts on the DVD corresponding to each sample is shown in Figure 2.6b. One can clearly see that the peak heights decrease proportionally to hCG standard concentrations of 25 mIU/mL to 0 mIU/mL (No.1-7). For the purpose of direct comparison, the ODR (as described above) of each binding strip was also determined. Figure 2.7a shows that both PIF density and ODR increase monotonically as a function of the hCG concentration; the two sets of data match well, but the PIF determinations show much lower background signal. In Figure 2.7b, the ODR is plotted as a function of the PIF density for each of the tested standard solutions. The linear relationship (except for the blank) illustrates a direct correlation between ODR and PIF. The ODR and PIF results confirm the accuracy of digital error reading and constitute a validated calibration curve for the analysis of unknown samples.

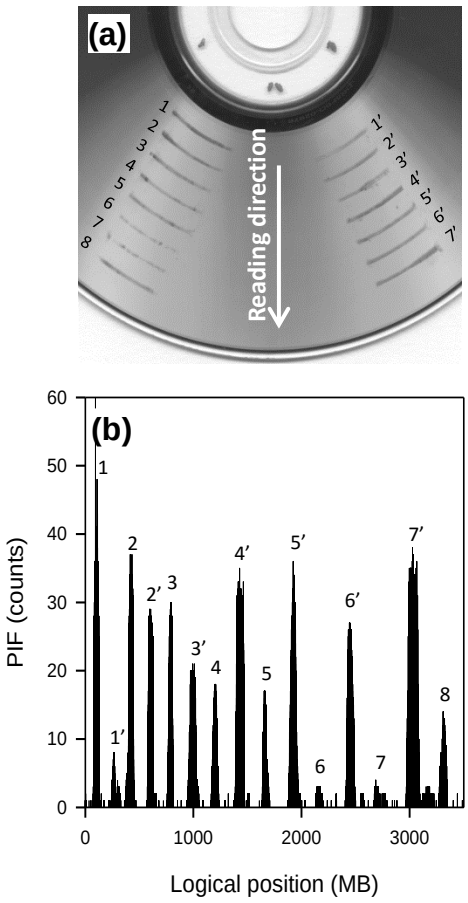

Figure 2.6. **Quantitation of hCG binding assays on a DVD. (a) Optical image shows a representative hCG DVDassay: hCG standards (1-7) and normal urine sample (8) are on the left-hand side of the picture, and the urine samples (1'-7') from different pregnant women are on the right-hand side. (b) Digital reading results of the DVDassay showing in (a), e.g., distribution of PIF peaks as function of the logical position on the DVD. Reproduced with permission from reference 59. Copyright © 2014, Royal Society of Chemistry.**

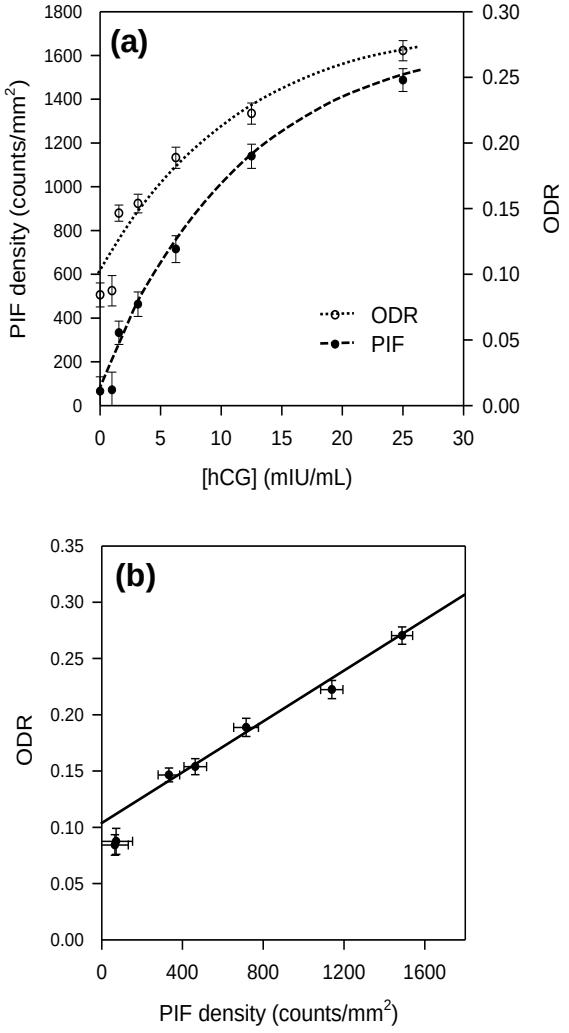

Figure 2.7. (a) Dependence of the PIF density and ODR on the concentration of hCG standard solutions. The dashed and dotted lines are to guide the eyes only. (b) Correlation of PIF density and ODR for the standard hCG solutions tested in (a). The error bars shown in both plots are standard deviations determined from three measurements of three independently prepared hCG DVDassays. Reproduced with permission from reference 59. Copyright © 2014, Royal Society of Chemistry.

After validation of the calibration curve, we tested and analyzed urine samples from different pregnant women after 31 to 38 weeks of their gestation. In this period of pregnancy, the hCG level should be elevated and remain relatively constant. This set of tests was included in the design shown in Figure 2.6a on the right-hand side (No. 1'-7');

the distribution of PIF counts on the DVD corresponding to each sample is also shown in Figure 2.6b. In order to examine the reliability of hCG quantitation with DVDassays, we also carried out a conventional quantitative ELISA test to determine the hCG concentrations of the same set of samples. Figure 2.8a shows that hCG levels analyzed by PIF density quantitatively agree with the results of conventional quantitative ELISA tests. The hCG concentrations obtained by both methods were within the values reported in the literature (615-17419 mIU/mL for the 27th to 40th week of gestation).[76] As a note, these urine samples had to be diluted (1000 to 7500 times) because of their relatively high concentrations of hCG. No obvious interference with either the background or the signal enhancement step was observed.

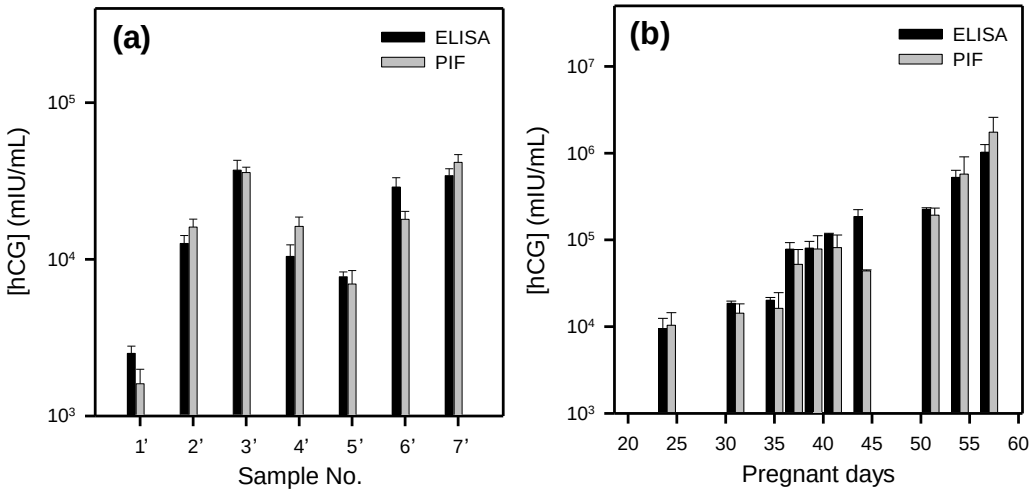

Figure 2.8. **(a) Quantitation of the urine hCG level of seven pregnant women after different periods (weeks) of gestation. (b) Quantitation of the urine hCG level of a pregnant woman after different periods (days) of pregnancy by two different methods (ELISA and PIF density). Four hCG DVDassays were prepared, but one of the test results is excluded as outlier due to degradation of hCG samples. The error bars shown in both plots are standard deviations determined from experimental results of three independently prepared hCG DVDassays. Reproduced with permission from reference 59. Copyright © 2014, Royal Society of Chemistry.**

Here, the most challenging task is to monitor the hCG level change of the same pregnant woman, which rise dramatically during the early stages of pregnancy for healthy

individuals. We therefore determined the hCG concentrations of a pregnant woman from the 24th to the 57th day of gestation by both the DVD-based assay and the conventional ELISA test. Figure 2.8b directly compares the two sets of data (PIF density and ELISA): the results were in agreement with the experimental uncertainties. As expected, a clear rising trend of the hCG level of the same woman during early pregnancy is observed with both methods.

In order to quantitatively evaluate the new diagnostic platform, the results obtained by the DVD-based assay (PIF density) versus the ELISA method were to compared data collected from different people (Figure 2.8a) and from the same patient (Figure 2.8b) at different pregnancy dates. Figure 2.9 shows a linear fit of these two sets of experimental data. The best fit line gives a slope of 1.06 ± 0.04, indicating that hCG levels measured by DVD assay are strictly consistent with those obtained by ELISA. The coefficient of determination (R^2) was calculated to be 0.98. This validates the DVD assay as a quantitative alternative to the current gold standard ELISA for biomedical analysis.

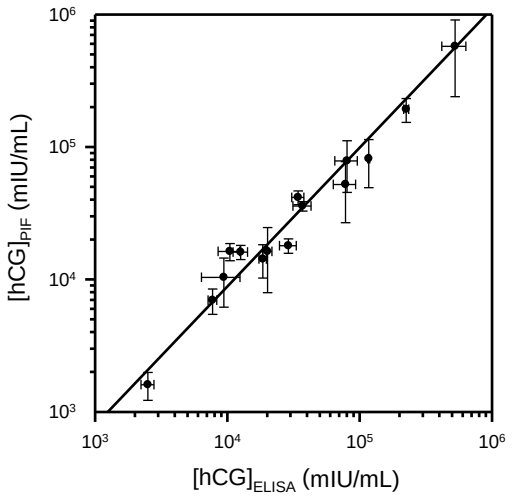

Figure 2.9. Correlation between the hCG concentrations determined by the DVD assay and by the ELISA from the data shown in Figure 2.8. The solid line shows the best linear fit to the experimental data. The error bars shown in the plot are standard deviations determined from three independently prepared hCG DVDassays. Reproduced with permission from reference 59. Copyright © 2014, Royal Society of Chemistry.

2.3.5. Specificity test and application perspective

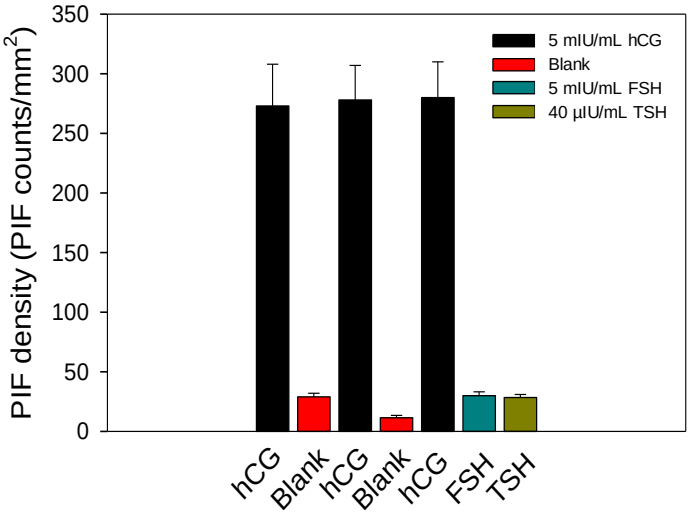

Figure 2.10. Specificity test of hCG DVDassays; FSH and TSH were used as negative controls (5 mIU/mL of FSH and 40 µIU/mL of TSH). The signals for the two negative controls (30 ± 10 counts /mm^2 and 29 ± 10 counts/mm^2) are only slightly higher than the blanks (buffer only). The error bars shown in the plot are standard deviations determined from three independently prepared DVDassays. Reproduced with permission from reference 59. Copyright © 2014, Royal Society of Chemistry.

Figure 2.10 shows a set of control experiments (FSH and TSH used as the interfering analyte), which confirms the specificity of the hCG-DVD assay. FSH is a hormone found in humans and other animals and the alpha subunits of both FSH and TSH are identical to those of hCG. With 5 mIU/mL FSH and 40 µIU/mL TSH (normal adult range is 0.3-5.0 µIU/mL)[77], the signals for the two negative controls (30 ± 10 counts/mm^2 and 29 ± 10 counts/mm^2) were only slightly larger than those for the blanks (Figure 2.10).

The presented hCG-DVD assay makes hCG quantitative tests at home with an ordinary optical drive or stand-alone DVD drive possible. The significance of hCG as a biomarker has been explored extensively in the past ten years. It has been discovered that hCG is in fact a group of molecules (regular hCG, hyperglycosylated hCG, and the free beta-subunit of hyperglycosylated hCG), each with a different function.[76,78] Beyond pregnancy detection, total hCG measurements and the detection of three hCG variants

can be important factors in monitoring pregnancy outcomes and determining the risk of Down syndrome fetus. An abnormal rise or decrease in the hCG level may also indicate preeclampsia or a placental site trophoblastic tumor or other human malignancies.

2.4. Conclusion

A DVD-technology based diagnostic platform was developed and evaluated by determining hCG levels in physiological buffers and in diluted urine samples of pregnant women. In particular, both qualitative and quantitative pregnancy tests can be performed on the same DVD (based on ODR determination and digital data reading error analysis), providing the advantages of commercially available pregnancy test strips and traditional ELISA kits. This platform will not only indicate pregnancy, but also provides a means to follow hCG levels through self-regulated monitoring. The hCG assays are carried out on standard DVD-Rs that are read with a standard computer drive in conjunction with free DVD-quality control software. As no modification of either the hardware or software driver is required, the DVD-based bioassay promises to be a platform technology for rapid, low-cost, and high-throughput point-of-care biomedical diagnosis.

Chapter 3.

Multiplex assay-on-a-BD for the quantitation of cardiac biomarkers*

3.1. Introduction

Optical disc technology has witnessed significant changes in the market share of digital data storage devices since the commercialization of USB flash drives in 2000.[80] Today, Blu-ray discs (BDs) appear to be the most popular media to store high-definition movies. Blu-ray technology is superior to CD and DVD recordings; owing to improved fabrication methods for disc media (see Chapter 1), the BD drive that utilizes a laser with a shorter wavelength (405 nm vs. 650 nm for DVD and 780 nm for CD, respectively), and an objective lens with larger numerical aperture (0.85 vs. 0.60 for DVD and 0.45 for CD).[1] The improved optics result in a much smaller laser focusing spot, which significantly increases the storage capacity (25 GB vs. 4.7 GB for DVD). Nevertheless, only limited studies have been reported in exploring BD technology for the development of analytical devices.[81] Arnadis-Chover *et al.* modified a Blu-ray drive by incorporating an external amplification/detection board for reading disc-based high-density DNA hybridization arrays and competitive immunoassays for microcystins.[82] Nieuwoudt *et al.* sputtered gold films on the nanostructured gratings of a BD to create inexpensive substrates for surface-enhanced Raman spectroscopy.[53] Donolato *et al.* converted a Blu-ray optical pick-up unit into an optomagnetic reading device to monitor the Brownian relaxation dynamics of modified magnetic nanoparticles in microfluidic devices for the quantitation of *E. coli* DNA.[39] Independently, Tian *et al*. reported Blu-ray technology-based optomagnetic reading of a competitive immunoassay for *Salmonella*.[83]

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*Reproduced in part from the manuscript "Blu-ray technology-based quantitative assays for cardiac markers: from disc activation to multiplexed detection", S. Weng, X. Li, B. Ge, M. Niu, and H.-Z. Yu, *Anal. Chem.* **2016**, 88, 6889–6896.[79] Copyright © 2016 American Chemical Society. S. Weng performed most of the experimental work; X. Li carried out the AFM studies, B. Ge and M. Niu provided ELISA data S. Weng and H.-Z. Yu wrote the manuscript.

We are interested in the development of digitized molecular detection systems by using unmodified optical drives to read various chemical and biochemical assays prepared on *bona fide* optical discs (CDs and DVDs).[2] Such a conceptually different approach has been validated and improved by others for cell counting[61] and for the detection of food allergens.[63] Herein, the possibility of creating a Blu-ray technology-based analytical platform with unprecedented sensitivity and resolution is explored. Beyond the search of new activation methods that work for the special coatings on BD discs, one must also identify an assay system that has practical applications either for on-site chemical analysis or for POC medical diagnosis. For the latter, we have decided to tackle the detection of cardiac biomarkers, as their on-time and accurate detection is crucial for diagnosis of acute myocardial infarction (AMI).

Cardiovascular diseases are the leading cause of death worldwide.[84,85] According to data reported from the World Health Organization and American Heart Association, globally 8.1 million people died from AMI, accounting for 1 in every 7 deaths worldwide in 2013.[86,87] Currently, AMI is diagnosed predominantly from any two combinations of determined AMI-relevant chest pain, related findings from electrocardiographic (ECG) examinations[88,89] and increased concentration of cardiac biomarkers, particularly myoglobin (MB) and cardiac troponin I or cardiac troponin T (cTnI or cTnI, respectively), and C-reactive protein (CRP). During AMI, the concentration of the respective biomarkers increases significantly with time.[88,89] The best solution is to test several qualitative cardiac biomarkers simultaneously to improve accuracy, sensitivity and specificity for the diagnosis of AMI and risk of reinfarction.

3.2. Experimental section

3.2.1. Materials and reagents

Standard samples of myoglobin (100 ng/mL), cardiac Troponin I (50 ng/mL), and their monoclonal antibodies were provided by Biogate Laboratories Ltd. (Burnaby, Canada). cTnI ELISA kits and cTnI serum samples were obtained from Vancouver Biotech Ltd. (Vancouver, Canada). A CRP standard sample (2.46 mg/mL) was purchased from Lee Biosolutions, Inc. (Maryland Heights, MO). The 72-mer synthetic oligonucleotide 5’-

NH_2-GGC AGG AAG ACA AAC ACG ATG GGG GGG TAT GAT TTG ATG TGG TTG TTG CAT GAT CGT GGT CTG TGG TGC TGT-3' was purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The biotin labeling kit-NH_2 was purchased from DOJINDO Molecular Technologies, Inc (Rockville, MD). The Sylgard 184 Silicone Elastomer kit was purchased from Dow Corning Corporation (Midland, MI). NHS, EDC, MES, BSA, and tween-20 were purchased from Sigma-Aldrich. Deionized water (>18.3 MΩ cm) was produced with a Barnstead Easy Pure UV/UF compact water system (Dubuque,IA).

The buffer solutions had the following compositions. Activation buffer: 0.1 M MES, and 0.9% NaCl, pH 5.8; immobilization buffer: 100 mM phosphate buffer, 150 mM NaCl, 5% glycerol, and 2 mM NaN_3, pH 7.4; blocking buffer: 100 mM phosphate buffer, 150 mM NaCl, 8% BSA, and 2 mM NaN_3 at pH 7.4; washing buffer: 100 mM phosphate buffer, 150 mM NaCl, 1% BSA, 0.1% gelatin, 0.05% tween-20, and 2 mM NaN_3 at pH 7.4; aptamer buffer: 100 mM phosphate buffer, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 2 mM NaN_3 at pH 7.4.

3.2.2. Surface activation and assay preparation

High-definition (HD) video files were burned on a blank BD-R (Verbatim 25 GB 6 x) that was then cleaned by rinsing with ethanol and deionized water. For the surface activation, the BD-R was immersed in a NaOH solution (0.1 M, 55°C) in a glass petri dish for 1.5 h. When taking out the treated BD from the petri dish, caution should be taken to avoid peeling off the label layer and to minimize undesired scratches to the disc surface.

The surface density of carboxyl groups presented upon hydrolysis on a BD-R surface was determined to assess the disc activation efficiency. For this purpose, a piece of hydrolyzed BD-R (3 cm × 3 cm) was immersed in a crystal violet solution (1 mM) for 5 min and then rinsed with deionized water. Bounded dyes were removed from the surface by two separate incubations (10 min each) with 80% ethanol (2 mL) and 0.02N HCl (2 mL), respectively. After the incubations, the remaining solutions were collected and combined in a conical tube. To determine the surface density of carboxyl groups, the solution in the conical tube was analyzed with Ocean Optics Mikropack DH-2000-BAL

spectrometer (Dunedin, FL, USA) by measuring its absorbance at 590 nm. The concentration of crystal violet was then calculated from Beer's law. The molar extinction coefficient (ε) of crystal violet is 100,000 M^{-1} cm^{-1}.[90]

A PDMS plate with three sets of microchannels (500 μm × 50 μm × 15 mm) was used to deliver the samples for the construction of the assay. Before binding the capture antibodies, 1 μL of 100 mM EDC and 25 mM NHS in the activation buffer were injected into each channel and incubated at room temperature for 3 h. Vacuum suction was applied to remove the solution in the microfluidic channels; then the channels were washed with deionized water.

To construct the MB and cTnI sandwich assays, anti-MB Mab (50 μg/mL, 1 μL/channel) and anti-cTnI Mab (50 μg/mL, 1 μL/channel) in the immobilization buffer were injected into different sets of channels and incubated for 1 h (to immobilize the capture antibodies onto the BD surface). The channels were then cleared by vacuum suction and washed with deionized water. The surface was then blocked by blocking buffer (1 μL/channel) for 2.5 h. The cardiac marker samples were diluted in the immobilization buffer, injected into the channels (1 μL/channel) and incubated for 1 h. Biotin-labeled anti-MB Mab (0.5 μg/mL) and biotin-labeled anti-cTnI Mab (0.5 μg/mL) in the washing buffer were then added into the channels (1 μL/channel) and kept for 1 h. The BD was rinsed with the washing buffer twice, followed by a signal amplification step.

For the preparation of CRP aptamer/antibody hybrid immunoassay, the biotinylated CRP aptamer solution (diluted in aptamer buffer, 0.1 μM, 1 μL/channel) was heated to 95°C and cooled quickly (ice bath) before addition to the surface. The capture antibody in this system was anti-CRP Mab at the concentration of 50 μg/mL.

3.2.3. Signal amplification and digital reading

Nanogold-streptavidin conjugates (0.8 μg/mL) in washing buffer were injected into the microfluidic channels (1 μL/channel) and kept for 50 min at room temperature. The BD was then rinsed with washing buffer (2 × 5 min) and deionized water (5 min). Finally, the PDMS plate was removed from the BD surface, and silver enhancement solution was introduced. It should be noted that the silver staining duration can be varied depends on

the preferred assay response range. The signal-enhanced BD was washed with deionized water and dried under N_2.

The BD assays were read with a conventional optical drive (PLEXTOR PX-LB950UE external Blu-ray) coupled with free disc-quality diagnostic software (PlexUTILITIES 1.3.3.1, http://www.goplextor.com/) to collect and determine the error distributions (in both LDC and BIS modes). The reading speed was set at 8 x to scan the entire disc within 10 min. The raw data was exported to Microsoft Excel or Sigma Plot to integrate the BIS counts for each testing site and determine the BIS density.

3.2.4. Infrared spectroscopy surface characterization of Blu-ray disc

The hard coating structure was analyzed by a Spectrum Two™ FTIR Spectrometer with an additional Universal Attenuated Total Reflectance (ATR) accessory (by PerkinElmer). Before the sample was placed onto the ATR top-plate, the background was collected. Once the hard coating layer was placed on top of the crystal area on the platform, the pressure arm was used to lock the sample at the center (crystal/sample area). Enough force was then applied to the sample, pushing it onto the diamond surface until the bands in the spectrum (in Preview Mode) could be clearly distinguished. The data was then collected after the adjustment. It should be noted that the BD hard coating layer was physically peeled off with a penknife.

3.2.5. ELISA validation

cTnI ELISA kits were provided by Vancouver Biotech Ltd. (Vancouver, Canada). A 96-well ELISA plate was pre-coated with four anti-cTnI monoclonal antibodies. Both the ELISA plate and the cTnI standard (50 ng/mL) were stored at -20 °C. All other reagents (cTnI zero standard buffer, enzyme conjugate reagent, and TMB reagent) were refrigerated at 2 to 8 °C. The cTnI standard set (12.5, 6.25, 3.13, 1.56, 0.78, 0.39, and 0 ng/mL) and unknown samples (50 µL/well) were then dispensed into the wells, followed by addition of the enzyme conjugate reagent (monoclonal anti-cTnI antibodies conjugated to horseradish peroxidase, 50 µL/well) and incubated for 1 h at room temperature. Afterwards the solution was removed and the wells were washed 5 times with 300 µL of 1

x wash buffer (diluted from 10 x wash buffer with deionized water). The TMB solution was introduced to the wells and incubated in the dark for 15 min at room temperature. Then, the 2N HCl was added into each well to stop the reaction (50 µL/well). The absorbance at 450 nm was then determined with an Infinite 200 PRO microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

3.3. Results and discussion

3.3.1. Digital reading protocol for “ink assays” on a BD

The storage capacity of a BD-R is 25 GB and we recorded HD video files that typically have an average size of 8 GB for movies of 2 hours length for testing the assay reading protocol described below. When reading the disc with recorded video files, errors may occur due to the noise arising from disc tilting, laser defocusing during the burning process or even from scratches, dust, and fingerprints left on the disc surface. Since the BD reading system is very sensitive to small particles/minor scratches on the surface, the structure of BD ECC cluster has been improved by inserting picket columns between the user data in order to provide better error correction capability. LDC and BIS are the two error testing modes used in the BD-quality diagnostic software to construct error distribution diagrams and indicate the number of reading errors shown.

To start with, we examined the disc after the recording process by using the free PlexUTILITIES 1.3.3.1 program. This software can determine the LDC and BIS error counts for every 0.0625 megabyte of data, which is the size of an ECC block in the Blu-ray system. Particularly, 21 ink stains of different sizes were drawn using a Pilot extra-fine metallic ink marker on a BD disc with previously recorded data (Figure 3.1a). As shown in Figure 3.1b, the BIS error reading yielded 21 distinct peaks with varied heights and widths. The highlighted four peaks correspond to the dots magnified in the inset of Figure 3.1a. Generally speaking, the peak sizes correlate with the dimensions of the ink dots, which have diameters from 96 to 605 μm (measured from Motic Image Plus 2.0ML). The smallest ink stain (< 100 μm in diameter) located at the far left is almost invisible in the photo; rather remarkably, the error reading program can produce a distinct peak. This result confirms

that a BD has a much better lateral resolution for identifying “ink assays” than a CD (~250 μm)[55] or a DVD (~180 μm).[59]

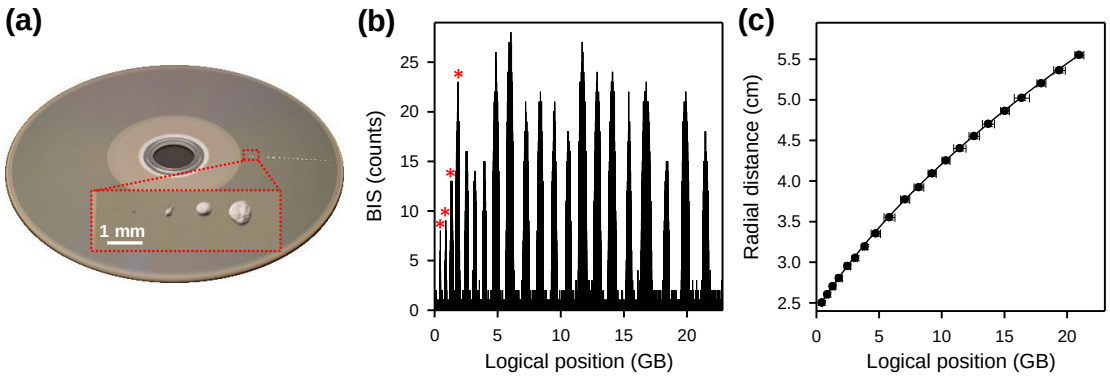

Figure 3.1. (a) Optical image of a BD-R with a series of 21 ink stains of different sizes. The inset is an enlarged view of the first four ink stains; (b) Error reading plot in the BIS mode of the BD-R with ink stains shown in (a). The red stars highlight the error peaks that correspond to the first four ink spots; (c) Correlation between the logical position and radial distance of the ink stains on the BD-R. The solid line is the best fit based on equation 3. The error bars shown in (c) are standard deviations determined from three repeated measurements of the peak positions on the same BD. Reproduced with permission from reference 79. Copyright © 2016, American Chemical Society.

To physically locate each of the ink stains, the radial distances of the ink spots (measured from the center of the disc to the center of each stain) are plotted as a function of the logical position of the error peak. As depicted in Figure 3.1c, the correlation between the two (radial distance and logical position) is fit by the following equation

$$r = \sqrt{\frac{x}{23.3} \times (5.8^2 - 2.4^2) + 2.4^2} \qquad (4)$$

where r is the radial distance on a BD (cm) and x is the corresponding logical position (GB). A single-layered BD-R has the capacity of 23.3 GB (GiB) and its outer and inner radius are 5.8 cm and 2.4 cm, respectively.[1] With this equation, one can readily determine the physical locations of an assay site if the logical position (with the pre-recorded data) is known. For example, the last error peak located at the logical position of 21.6 GB is produced by the ink stain located at 5.62 cm from the center of the disc.

3.3.2. Surface activation and preparation of assay-on-a-BD

There is a special hard coating material on BD discs, so we explored different surface activation methods other than the UV/ozone treatment that was successful for the CD/DVD surface (polycarbonate substrate).[55,59] The hard coating material on a BD-R is typically an UV-curable resin with dispersed silica nanoparticles.[26] Before performing any surface activation, we examined the hard coating material of BD-Rs using energy-dispersive X-ray spectroscopy (EDS) and Infrared spectroscopy (IR), and confirmed that the featured Hard Coat™ of Verbatim BD-Rs is, in fact, based on lactone composites.[22] As shown in Figure 3.2, no peaks related to silicon (1.74 keV) can be identified. This result indicates that the surface coating of the Verbatim BD-Rs used in this work is not the typical composite material of UV-curable acrylic resin and nanoparticles of silica. We further studied the composition of the Hard Coat™ layer of Verbatim BD-Rs using Infrared spectroscopy. The spectrum shown in Figure 3.3 was obtained on a Spectrum Two™ FTIR Spectrometer with an Attenuated Total Reflectance (ATR) accessory (PerkinElmer). The two distinct peaks at 1726 cm^{-1} (C=O stretching) and 1150 cm^{-1} (C-O stretching),[91] indicating that the hard coating material on Verbatim BD-Rs is a (meth)acrylate-based lactone composite.[92]

A common base, sodium hydroxide (0.1 M), has been used to perform a hydrolysis reaction (90 min) on BDs at an elevated temperature (55 °C); the surface was characterized by measuring the values of contact angles using a set of buffer solutions (pH 2 to 12). In addition, no corrosion of the surface was observed after the activation. In other words, the treated BDs are still readable by an unmodified optical drive.

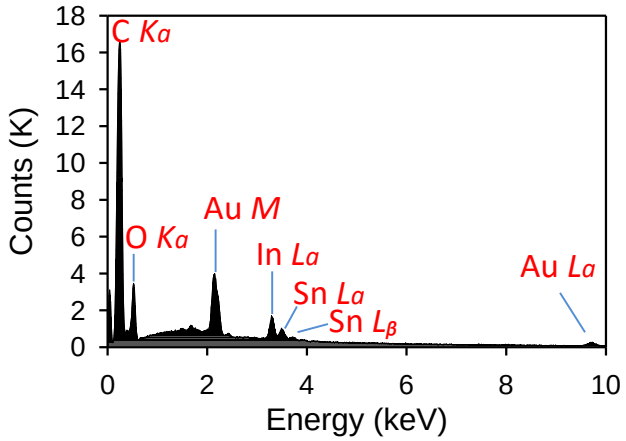

Figure 3.2. **EDS spectrum of gold sputtered hard coating layer on Blu-ray disc. No significant peaks related to silicon (1.74 keV) can be identified. The carbon and oxygen peaks appear very strong. The peaks at 2.12 and 9.71 keV are from the sputtered gold film, which may not be present originally. The indium peak (3.28 KeV) and tin peaks (3.44 KeV and 3.66 KeV) indicate that conductive materials (presumably indium tin oxide) are incorporated to the coating layer. All peaks were assigned based on reference 93. Reproduced with permission from reference 79. Copyright © 2016, American Chemical Society.**

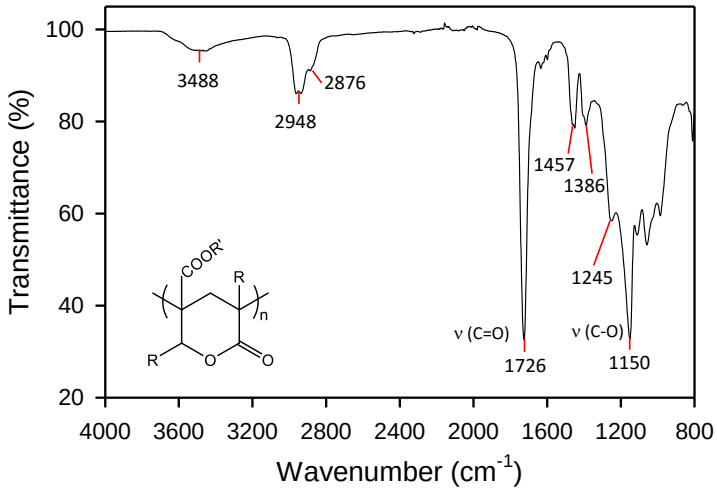

Figure 3.3. **ATR-IR spectrum of the Hard Coat™ film of Verbatim BD-Rs. The peaks were assigned based on reference 92. Reproduced with permission from reference 79. Copyright © 2016, American Chemical Society.**

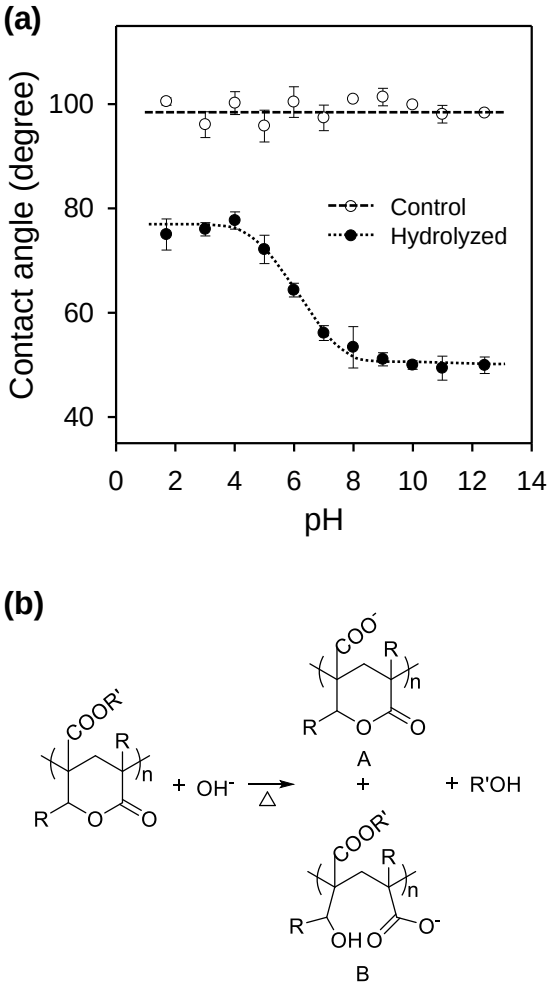

Figure 3.4. **(a) Contact angles of buffer solutions on a BD-R surface treated with 0.1 M NaOH for 1.5 h at 55°C (solid circles) and an untreated BD-R surface (open circles). The transition between pH 4 and 9 confirms the formation of reactive -COOH groups. The dashed and dotted lines are to guide the eyes only. Each data point represents the average reading of at least three replicated measurements, and the error bars show the corresponding standard deviation. The error bars shown in the plot are standard deviations determined from three measurements of the same BD. (b) Possible hydrolysis reactions on the BD-R surface. Reproduced with permission from reference 79. Copyright © 2016, American Chemical Society.**

As shown in Figure 3.4a, the contact angle remains at 98.9 ± 1.6° on the untreated surface throughout; in contrast, the contact angles of the hydrolyzed BD surface show a clear transition (78 ± 2° to 51 ± 2°) at pH 4 to 9. This transition in surface hydrophilicity indicates that reactive carboxyl groups were generated on the surface. The extracted pK_a

(~6) is comparable with that obtained on carboxy-terminated self-assembled monolayers on gold.[94] It has been observed previously that the pK_a value on the surface is typically 2-4 pH units higher than that of the solution pK_a (3-5).[95] Such a difference reflects the increased difficulty of generating the charged form of functional groups (from –COOH to $–COO^-$) at the solid/liquid interface.[96] The surface density of thus generated carboxyl groups was determined to be $1.1 \pm 0.1 \times 10^{-8}$ mol/cm^2 on a BD surface, which is much higher than that on the surface of CDs/DVDs ($4.8 \pm 0.2 \times 10^{-10}$ mol/cm^2).[71]

The challenging question is determining which reactions have occurred on the surface upon hydrolysis. Figure 3.4b shows the two possible products (A, and B) that could be formed in the (meth)acrylate-based lactone coating are listed.[97] The first product comes from the hydrolysis of ester groups on the sidechain, while another results from opening of the lactone ring via acyl-oxygen cleavage.[98,99] To identify which product are the most favorable, several factors such as polymer structure and temperature need to be considered, which is beyond the scope of this thesis. Most importantly, the generation of reactive carboxyl groups on the BD surface provides a viable means to covalently attach probe molecules (via amide coupling) for creating the bioassays of interest.

Maquieira and co-workers have explored the preparation of bioassays on the BD surface based on physioadsorption, i.e., either streptavidin (for immobilizing biotinylated DNA strands) or a coating conjugate (BSA-MC, for the detection of microcystin LR) was spotted on the surface using a contact liquid dispenser or a non-contact arrayer.[82] They improved the immobilization strategy by spin-coating a thin layer of SU-8 to which thiolated oligonucleotides were attached via a thiol-epoxy coupling reaction.[100] In this study we directly immobilize the capture antibodies on the BD surface via amide-coupling upon the activation of the surface carboxyl groups with EDC and NHS.

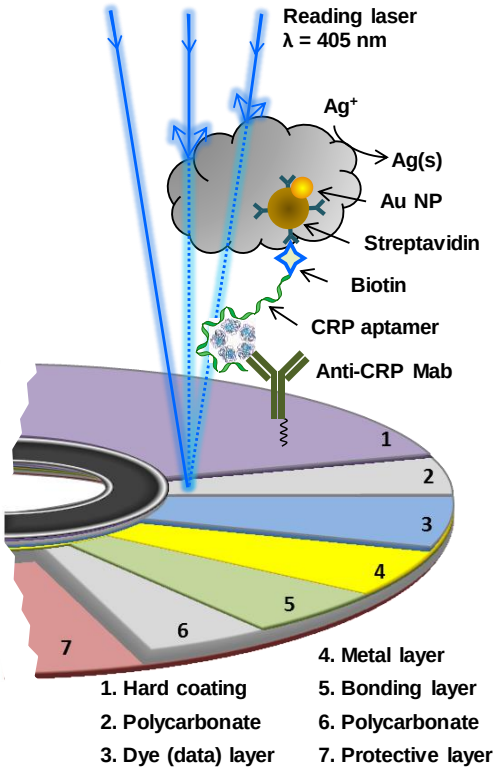

Figure 3.5. Schematic view of the configuration of an assay-on-a-BD (an aptamer-antibody hybrid assay for CRP, for example). The silver particles formed at binding sites would interfere with the laser reading, which induces significant errors upon reading with a standard Blu-ray drive. Note that the aptamer-CRP binding shown here is not the actual folded structure, but a graphical representation. Reproduced with permission from reference 79. Copyright © 2016, American Chemical Society.

As shown in Figure 3.5, the analyte was introduced to the assay surface after the successful probe antibody immobilization. Different from the standard immunoassays for MB and cTnI, we have prepared an aptamer-antibody hybrid assay for the detection of CRP (Figure 3.5). The CRP-specific aptamer (a 72-mer oligonucleotide) was previously selected and characterized by Huang *et al.*[101] The 5'-end of this aptamer is labeled with biotin and the introduction of nanogold-streptavidin conjugates facilitates the subsequent signal enhancement step (i.e., silver staining). Since biomolecules are too small (<<1/4 λ, λ = 405 nm for BD) to induce a significant laser disruption when read with an optical drive, a silver enhancement step (gold nanoparticle-promoted reduction of Ag^+) is necessary for signal amplification. As the commercial Nanoprobes LI silver enhancement kit requires a

longer staining time (at least 30 min) to obtain a readable signal, we optimized the composition and concentration of the silver salt and reducing agent solutions to achieve a faster assaying process (within 10 min).

3.3.3. Multiplex assay-on-a-BD for cardiac markers

The three cardiac markers CRP, MB, and cTnI were specifically chosen because of their importance for the diagnosis of cardiovascular diseases. All three are released in human serum after the heart damage (MB: 1 to 3 h, cTnI: 3 to 12 h, and CRP 4 to 6 h after AMI)[102,103], they are ideal markers for early AMI screening. Due to the low specificity of MB and CRP, a multiplexed detection of all three would be most favorable.[89]

As shown in Figure 3.6, with the aid of a PDMS channel plate, binding assays for all three marker proteins were prepared on the same BD. The PDMS plate has three sets (21 in total) of embedded microfluidic channels, which were used throughout the entire process for assay alignment and minimizing sample volume (1 µL per channel). Particularly MB assays (1-7: 0-100 ng/mL) are on the left side, cTnI assays (1'-7': 0-1.6 ng/mL) in the middle, and CRP (1"-7": 0-10 ng/mL) on the right side. The corresponding concentrations for individual assays, as discussed below, were chosen based on clinical needs, their abundance under normal conditions, and their abundance at the time of having an AMI. In the scanned image of the assay disc (Figure 3.6b), the background appeared dark, the assay lines were brighter, demonstrating that with higher concentrations of the analyte, the assay lines became more distinct. As shown in Figure 3.7, besides the high background, the signal changes for the entire concentration range are small (from 60 to about 100). On the other hand, the error reading plot shows unequivocal quantitative results (Figure 3.6c). One can see clearly that in all three assays that with increased concentrations of the marker protein a higher (more distinct) error peak was generated. In all cases the background signals, labeled as 7,7',7", were negligible.

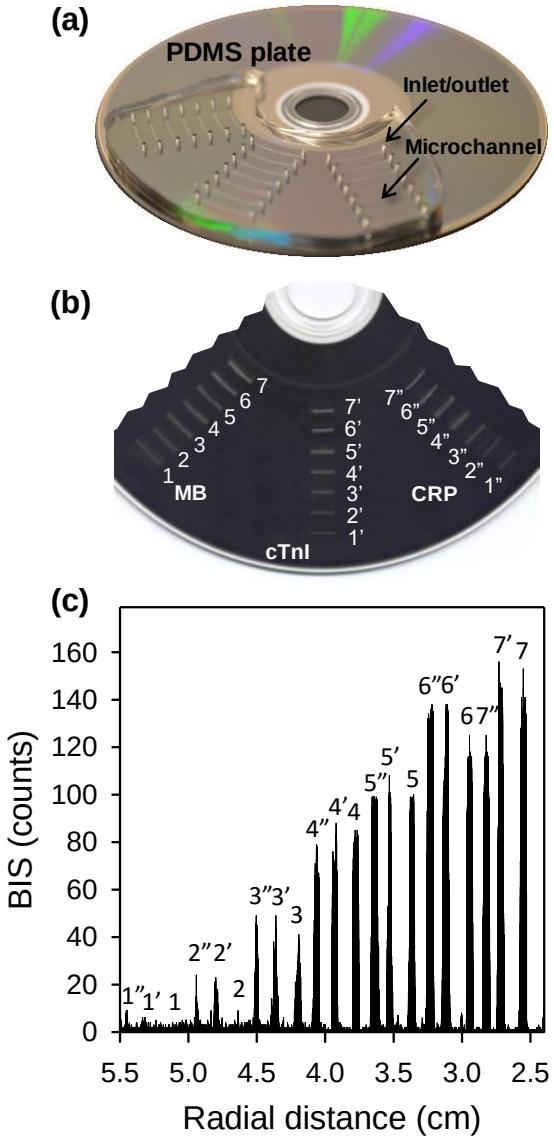

Figure 3.6. **Assay-on-a BD for the multiplex detection of cardiac biomarkers. (a) Photo of the PDMS channel plate affixed to the BD surface for constructing the bioassays; (b) Scanned image showing assays with different concentrations of the three marker proteins; MB assay (1-7), cTnI assay (1'-7'), and CRP assay (1"-7"); (c) Error distribution plot of the assays shown in (b) upon reading in a standard Blu-ray drive. Reproduced with permission from reference 79. Copyright © 2016, American Chemical Society.**

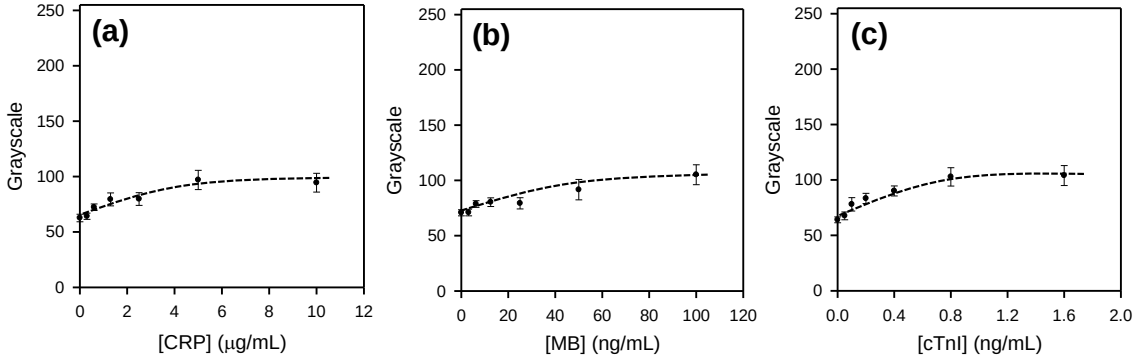

Figure 3.7. Dependence of the grayscale intensity on the concentration of (a) CRP, (b) MB, and (c) cTnI. The data were determined from the grayscale image shown in Figure 3.6b of the main text. Four cardiac biomarkers assays-on-a-BD were prepared, but only three of them shows satisfactory results. The error bars shown in the plots are standard deviations determined from experimental results of three BDassays. Reproduced with permission from reference 79. Copyright © 2016, American Chemical Society.

The American Heart Association and the Center for Disease Control and Prevention define people as high risk if their CRP concentrations exceed 3.0 μg/mL.[88] In general, commercially available CRP assays cover the 0.5 to 10 μg/mL concentration range.[103] Figure 3.8a shows the CRP assay-on-BD tested in the same range. Initially the assay signal (BIS density, i.e., BIS errors per unit area) increases with the CRP concentration. At higher concentrations (> 5 μg/mL), the signal becomes saturated. In the red region, the aptamer/antibody hybrid CRP assay we built on BD adequately determines the threshold concentration. For MB, the normal serum concentration for a healthy individual is typically in the range of 19 to 92 ng/mL (males) or 12 to 76 ng/mL (females).[104] The MB assay-on-BD covers the range up to 100 ng/mL (Figure 3.8b) such that elevated concentrations can be easily tested as well. In comparison with CRP and MB, the detection of cTnI is much more challenging, because the upper reference limit is very low (0.08 ng/mL) for a healthy person, although it would increase dramatically for myocardial infraction patients (up to 100 ng/mL).[89] Figure 3.8c shows that the cTnI assay-on-BD can test the 0.05 to 1.6 ng/mL range. The threshold concentration is on the low side, but with further optimization of the antibody pairs and assay conditions, one should be able to create a quantitative assay in this ultralow concentration range. In comparison with the

colorimetric method, the BDassay shows two to three orders of magnitude higher signals using the error-reading protocol.

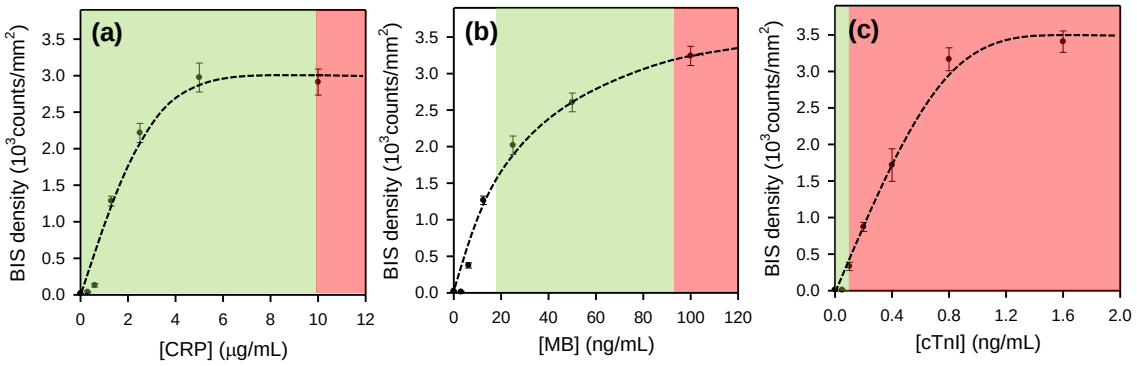

Figure 3.8. Dependence of BIS density on the concentration of individual protein biomarkers. (a) CRP; (b) MB; (c) cTnI. The green region indicates the range for healthy individuals, the red region indicates the abnormal range. The dashed lines are to guide the eyes only. Four cardiac biomarkers assays-on-a-BD were prepared, but only three of them shows satisfactory results. The error bars shown in the plots are standard deviations determined from the experimental results of three BDassays. Reproduced with permission from reference 79. Copyright © 2016, American Chemical Society.

To further understand the correlation between assay signal and analyte concentration, cTnI assays on a BD-R were examined by scanning electron microscopy (SEM). The SEM images (Figure 3.9) reveal that the density of silver deposits on the surface increases gradually with increasing cTnI concentration, while the particle size remains unchanged (~200 nm diameter). Analysis of the surface coverage of the silver "islands" shows a good correlation with the observed error density. The assay with 0.1 ng/mL shows coverage is as low as 0.6 ± 0.3% and up to 84.9 ± 1.6% when the concentration of cTnI is increased to 1.6 ng/mL (Table 3.1). The high-resolution image shown in Figure 3.9(f) shows that the surfaces are in fact covered with clusters (150-300 nm) of much smaller nanoparticles (20-70 nm in diameter). In fact, the particle sizes are much smaller than those of assays on the CD/DVD surfaces (~90-300 nm).[55] The particle size would decrease with a higher number of seeding sites for a given concentration of silver ions due to the silver ion competition of growing sites[105,106] so the much higher

surface density of carboxyl groups generated on the BD surface (compared to CD and DVD) may account for more efficient silver deposition (in turn a better assay sensitivity).

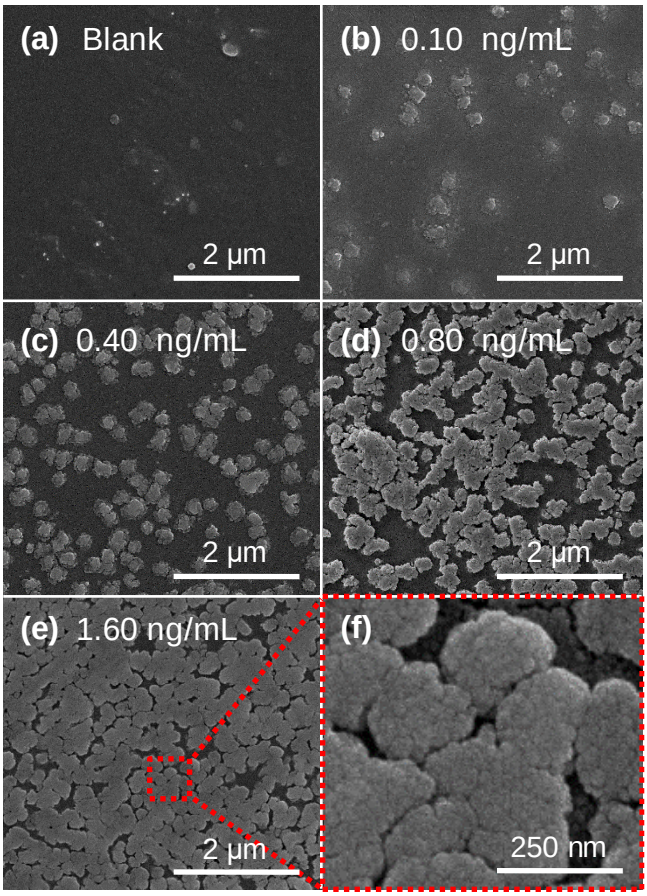

Figure 3.9. **SEM images of the cTnI assay-on-a-BD. Images (a) to (e) correspond to the binding strips on the BD-R with blank, 0.10, 0.40, 0.80, and 1.60 ng/mL of cTnI, respectively. The image (f) shows an enlarged view of the binding strip with a concentration of 1.6 ng/mL. Reproduced with permission from reference 79. Copyright © 2016, American Chemical Society.**

Table 3.1. **Correlation of surface coverage of silver particles, BIS density, and grayscale intensity of the binding strips in the cTnI assay.[79] The associated uncertainties are standard deviations determined from three BDassays.**

[cTnI] (ng/mL)	Surface coverage (%)	BIS density (10^3 counts/mm^2)	Grayscale intensity
Blank	0.6 ± 0.3	0.01 ± 0.01	64.0 ± 2.6
0.10	6.4 ± 2.0	0.33 ± 0.06	78.0 ± 6.0
0.40	28.8 ± 1.1	1.72 ± 0.22	90.1 ± 4.5
0.80	57.1 ± 2.4	3.17 ± 0.16	102.8 ± 8.3
1.60	84.9 ± 1.6	3.41 ± 0.15	104.0 ± 9.0

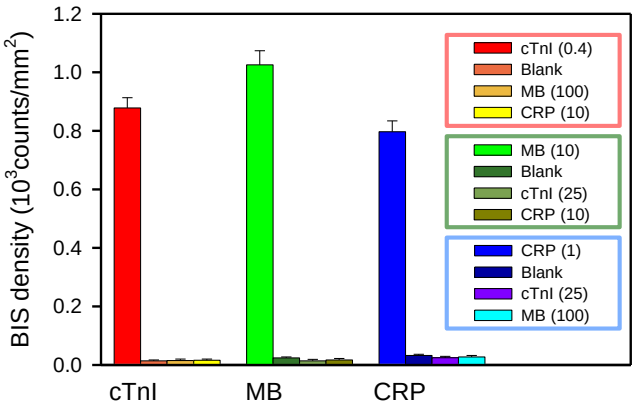

Figure 3.10. **Specificity test for the multiplexed cardiac assays on a BD; the concentration of corresponding targets is 0.4 ng/mL of cTnI, 10 ng/mL of MB, and 1 µg/mL of CRP; the negative controls are the two unrelated cardiac markers with 25 ng/mL cTnI, 100 ng/mL MB, and 10 µg/mL CRP. The numbers shown in brackets are concentrations in µg/mL. The error bars are standard deviations determined from three BDassays. Reprinted with permission from reference 79. Copyright © 2016, American Chemical Society.**

3.3.4. Real sample test and ELISA validation

Since cTnI is the most important cardiac marker for early diagnosis of AMI in emergency patients, the reliability of BD assays relative to conventional ELISA was evaluated by analyzing the same set of serum samples from an AMI patient to determine

the cTnI concentrations. As mentioned above, the concentration of cTnI rises rapidly when AMI occurs. As such, the cTnI testing range on the BD was extended to 0 to 12.5 ng/mL based on the initial ELISA testing results. One of the advantages of the silver enhancement strategy is the ability of tuning the response range, as the silver staining time can be shortened if one wishes to adjust it to a higher saturation concentration. As shown in Figure 3.11a, on the same disc we tested both cTnI standards (test strips 1-7) and serum sample replicates (S1-S5). It should be noted that S1 and S4 were tested at different dilutions due to the relatively high initial cTnI concentration in the original sample. The error reading histogram of the cTnI assay is presented in Figure 3.11b. The determination of the cTnI concentrations in the unknown samples is based on the calibration curve shown in Figure 3.11c, with a linear range up to 6 ng/mL and an R^2 value of 0.996.

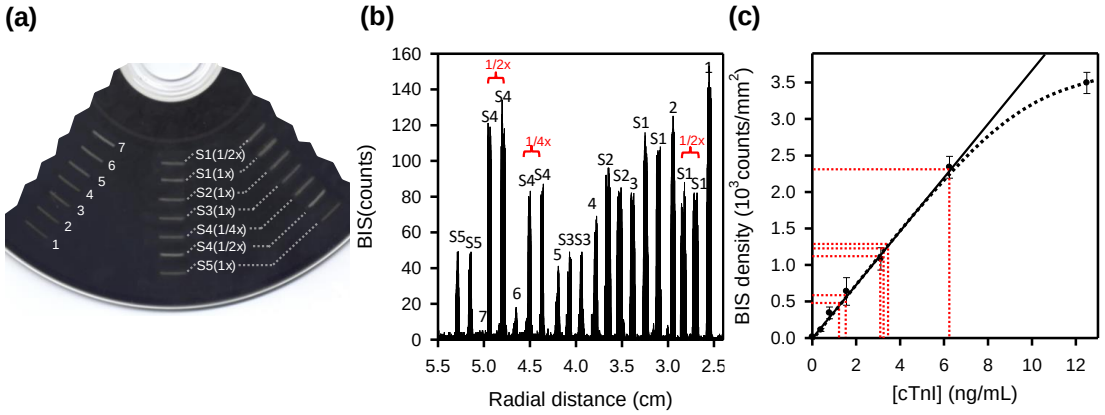

Figure 3.11. Quantitative cTnI assay on a BD-R. (a) Optical image showing the assay design: cTnI standards (1-7) are on the left-hand side of the picture, and two sample duplicates S1-S5 are in the middle and on the right side of the picture. The tests on S1, same as S4, are done with different dilutions; (b) Error-reading histogram of the cTnI assay-on-a-BD; (c) Dependence of BIS density on the concentration of cTnI standards (black circles). The red dotted lines indicate the corresponding signals and concentrations of unknown samples and the solid line is the best fit to the experimental data in the linear response region. The dotted line is to guide the eyes only. Three cTnI BDassays were prepared and all of them show satisfactory results. The error bars in (c) are standard deviations determined from three measurements of the same BDassay shown in (a). Reprinted with permission from reference 79. Copyright © 2016, American Chemical Society.

Figure 3.12 shows the correlation of cTnI concentrations obtained from assay-on-BD assays and commercial ELISA kits. Both the unknown samples determined in Figure 3.11c and standards were included. The linear fit to the experimental data has a slope of 1.03 ± 0.04 with an R^2 value of 0.983. The close-to-unity slope and R^2 value confirm the reliability of the newly developed BD assays in the quantitation of protein biomarkers.

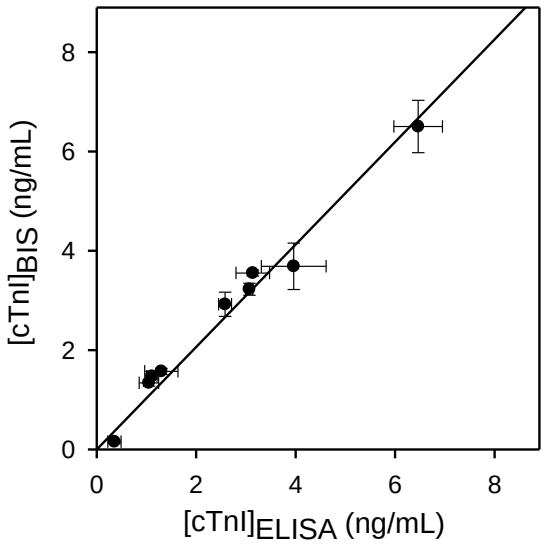

Figure 3.12. Correlation between the cTnI concentrations determined by Blu-ray assay and traditional ELISA from all cTnI samples. The solid line indicates the best linear fit to the experimental data. The error bars in the plot are standard deviations determined from three BDassays and three cTnI ELISA tests. Reproduced with permission from reference 79. Copyright © 2016, American Chemical Society.

In comparison with the traditional ELISA assays, the advantages of assay-on-a-BD rely on the measurement portability, cost effectiveness, and time savings. In general, ELISA tests need to be performed in a laboratory or hospital setting with trained professionals and require large amounts (~200 µL per well) of samples and reagents. More importantly, the turnaround time is around two hours that is insufficient to make a quick decision on the treatment of patients who had an AMI earlier. In addition, specialized assay reading equipment (e.g., microplate reader) is needed, which costs typically more than$4,000 USD. For the assay-on-a-BD, only an inexpensive standard optical drive or disc player is required to read the assay, which are prepared on mass-produced BD-Rs.

Most importantly, the surface chemistry developed for activating the disc and enhancing the signal is mild and efficient, and can be easily adapted for other applications. We should emphasize that the cardiac marker detection presented here is a trial system to demonstrate the working principle of the Blu-ray technology-based digitized molecular diagnosis platform. Extensive studies are warranted to tackle the challenges in a real-world clinical setting. To establish a practical POC diagnosis platform, one may have to further shorten the assay time. One possibility is to eliminate the biotin/nanogold streptavidin labeling step (directly applying nanogold-antibody conjugates) or to search for a more efficient signal enhancement strategy.

3.4. Conclusion

BD-R surfaces can be activated via simple hydrolysis with base at slightly elevated temperature, which makes possible the development of BD technology-based bioassays for the multiplex detection of cardiac marker proteins. The assay can be constructed with either antibody pairs or DNA aptamer/antibody hybrid structures. Upon silver enhancement the assays can be read with a conventional Blu-ray optical drive using free quality-control software. The assay-on-a-BD approach provides wide concentration ranges and impressive detection limits for the protein biomarkers being tested. In addition, the assay-on-a-BD shows great quantitation capabilities, the results of which correlate well with standard ELISA tests. The dramatically reduced cost of instrumentation and sample consumption augments the potential of assay-on-a-BD as a user-friendly, and high-throughput analytical platform for point-of-care diagnosis and on-site chemical analysis.

Chapter 4.

General conclusions and future directions

4.1. Concluding remarks

In this thesis, the performance of disc-based diagnostic platforms, with a focus on adaptation of DVD and BD technologies, has been demonstrated for the quantitative detection of biomedically relevant molecular analytes. These diagnostic platforms take advantage of the elegant error-correction function embedded in the disc's data structure to integrate reading errors upon forming silver-stained binding strips on the disc surface as the signal readout. Particularly, it has been shown that hCG assays prepared on a DVD can perform both qualitative (similar as pregnancy test strips) and quantitative tests of clinical samples. Both the detection limit (1.5 mIU/mL) and dynamic response range (up to 25 mIU/mL) are comparable with the standard ELISA tests. The quantitation of hCG is important for monitoring abnormal pregnancy and diagnosing gestational trophoblastic diseases or testicular cancers as noted in earlier sections.

For the development of BD-technology based bioassays, it was shown that the hard coating of BD-Rs can be activated via a simple and mild hydrolysis reaction in base under elevated temperature. The surface activation produces high density of reactive carboxylic acid groups, yet the morphology and optical properties remain intact, which ensures the readability of the modified discs. This novel surface activation method for lactone composite materials may be widely used for the future development of other types of plastic-chip based bioassays.

The multiplex assay-on-a BD systems shows remarkable performance for the detection and quantitation of key cardiac marker proteins, MB, cTnI, and CRP, in particular. Not only these markers in their respectively physiological concentration range can be tested simultaneously on the same disc, but also the quantitation cTnI in serum samples shows unequivocal correlation with the ELISA data. The work described in my thesis augments the potential of employing DVD or BD-technology based bioassays to perform self-regulated monitoring at home or diagnosis in a doctor's office. It is necessary to

emphasize again the unique aspect of using *bona fide* discs and drives for molecular detection, i.e., no need to modify either the hardware (optical discs and drives) or the software driver, which ensures its future as a low-cost POC diagnostic tool for population based screening and testing.

4.2. Future directions

Since the DVD and BD technology-based detection platforms presented in this thesis are merely trial systems, more studies should be performed to improve their performance for practical applications. As the "*need for speed"* is a critical consideration in developing and applying any POC tools, the assay preparation time should be shortened. One possibility is to eliminate the biotin/nanogold streptavidin labeling step by using nanogold-antibody conjugates directly. Even with the current signal enhancement protocol, additional efforts to optimize the type of sliver salts, the choice of reductant, and assay time shall be made. By improving the PDMS stamp design for multiplex capability, we can also shorten the overall time needed for testing a large number of samples. Additionally, we can fabricate a special BD with embedded microfluidic channels to perform cells or microparticle counting. One of possibility is to follow the protocol developed by Liu and co-workers,[61] i.e., to make a trench that is deep enough to perfectly fit a PDMS chip in the disc.

Additional testing of serum and whole blood samples using the optical disc-based diagnosis platform is also necessary in order to investigate if it is practical for real-world application. There are many challenges to overcome due to the complex nature of serum samples, the test results may be influenced by various components in the sample that cause severe interference to the signal reading. Pre-treatment or purification of the sample (e.g., centrifugation) before introduction to the platform should be carefully considered. Other types of discs and portable disc players available in the market can also be investigated as new diagnostic platforms in the future. Particularly, commercially-available MiniDVDs have the potential to be pocket sized detection platforms, which minimize the size of the apparatus (Figure 4.1). Its data storage capacity (1.4 GB) is smaller than that of a regular DVD (4.7 GB), which might limit the number of samples being simultaneously analyzed. The performance and its reading mechanism of the MiniDVD also need to be

evaluated accordingly. In line with the software approach, a portable disc player has the potential to be converted into a stand-alone prototype diagnostic tool (Figure 4.2). A customized disc quality-control software that has more user-controlled parameters can be used to make the system more efficient and user friendly. For example, the user would be able to select the laser scanning region (region of interest) to be analyzed, shortening the total analysis time. This technology is a milestone in achieving quantitative detection and imaging with disc technology-based analytical devices, and that more success will be appearing in the near future.

Figure 4.1. Photo of a MiniDVD. It has a diameter of 80 mm and a center hole of 15 mm diameter with a thickness of 1.2 mm.

Figure 4.2. A portable DVD player that may be adapted as a stand-alone POC device.

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