Magnetic nanochain enabled microarray and microfluidic bioassays

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TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**MAGNETIC NANOCHAIN ENABLED MICROARRAY
AND MICROFLUIDIC BIOASSAYS**

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2019

Magnetic Nanochain Enabled Microarray and Microfluidic Bioassays

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A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Supervisor Declaration Statement

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29 March 2019

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Date

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Assoc. Prof. Hongwei Duan

Authorship Attribution Statement

This thesis contains material from 2 papers published in the following peer-reviewed journals in which I am listed as an author.

Chapter 3 and 5 is published as Qirong Xiong, Chun Yee Lim, Jinghua Ren, Jiajing Zhou, Kanyi Pu, Mary B. Chan-Park, Hui Mao, Yee Cheong Lam, Hongwei Duan. Magnetic nanochain integrated microfluidic biochips. *Nature Communications* **2018**, 9, 1743. DOI: 10.1038/s41467-018-04172-1

The contributions of the co-authors are as follows:

- I co-designed the study with Dr. Chun Yee Lim, Prof. Yee Cheong Lam and Prof. Hongwei Duan. I performed all the laboratory work at the School of Chemical and Biomedical Engineering.
- Dr. Chun Yee Lim assisted in the fabrication of microfluidic device and theoretical calculation.
- Prof. Jinghua Ren assisted in the collection of clinical samples and clinical data.
- Dr. Jiajing Zhou assisted in the characterization and testing of magnetic nanochains.
- I, Chun Yee Lim, Prof Yee Cheong Lam and Prof Hongwei Duan analyzed the data and wrote the manuscript.
- Prof. Kanyi Pu., Prof Mary B. Chan-Park and Prof Hui Mao discussed the results and commented on the manuscript.

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The contributions of the co-authors are as follows:

- Prof Hongwei Duan provided the initial project direction and provided the guidance of whole study.
- I performed all the laboratory work at the School of Chemical and Biomedical Engineering.
- Dr. An Eng Lim conducted the 2D numerical simulation of liquid mixing.
- I wrote the drafts of the manuscript. The manuscript was revised together with Dr. An Eng Lim, Yun Lim, Prof. Yee Cheong Lam and Prof. Hongwei Duan.

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Date

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Aug 19, 2018

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List of Abbreviations

PDA	Polydopamine
MiChip	Magnetic nanochain integrated biochip
SERS	Surface-enhanced Raman scattering
ELISA	Enzyme-linked immuno sorbent assay
AuNRs	Gold Nanorods
AuNPs	Gold Nanoparticles
QDs	Quantum dots
LSPR	Localized surface plasmon resonance
TRIS	Tris(hydroxymethyl)aminomethane
HS-PEG-COOH	thiolated caboxy-poly(ethylene) glycol
CTAB	Cetyltrimethylammonium bromide
mPEG-SH	Thiolated methoxy-poly(ethylene) glycol
SDS	Sodium dodecyl sulfate
MES	2-(N-Morpholino)ethanesulfonic
EDC	Ethyl dimethylaminopropyl carbodiimide
NHSS	Sulfo-Nhydroxysuccinimide
BSA	Bovine serum albumin
HAS	Human serum albumin
CTC	Circulating tumor cell
NTP	4-Nitrothiophenol
BTP	4-bromothiophenol
TFTP	2,3,5,6-tetrafluorothiophenol
DFTP	3,5-difluorothiophenol
DCTP	2,4-dichlorothiophenol
MATT	4-methoxy- α -toluenethiol
DMF	<i>N,N</i> -Dimethylmethanamide
PVP	Polyvinyl Pyrrolidone

SSC buffer	Saline-sodium citrate buffer
PBS	Phosphate-buffered saline
ssDNA	Single-stranded deoxyribonucleic acid
Cy5	Cyanine 5
FITC	Fluorescein isothiocyanate
PSA	Prostate specific antigen
CEA	Carcinoembryonic antigen
AFP	Alpha-fetoprotein
<i>E. coli</i> O157:H7	<i>Escherichia coli</i> O157:H7
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
2-D	Two dimensional
CV	Coefficient of variation
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TIRF	Total Internal Reflection Fluorescence

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Summary

Magnetic Nanochain Enabled Microarray and Microfluidic Bioassays

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There are growing demands for bioassay techniques that allow for rapid, multiplexed detection of a panel of targets with minimum specimen consumption. However, the devices of small size often present fundamental problems such as intrinsic poor liquid mixing and inefficient mass transfer across microfluidic channels and diffusion-limited on-surface assays, which create considerable obstacles to achieve rapid, ultrasensitive detection. Recently, we developed polydopamine (PDA) based strategy to produce one-dimensional (1-D) magnetic nanochains with rigid structure. The magnetic nanochains immediately align in a magnetic field and undergo synchronous rotation in response to a rotating magnetic field, making them ideal nanomixers to promote fast fluid mixing and analyte transport in microscale devices. Importantly, PDA not only serves as the scaffold to lock the nanochain structure, but also allows for convenient surface functionalization by means of spontaneous Michael addition and/or Schiff base reaction with nucleophilic thiol and amine groups. As such, the nanochains can be sequentially functionalized with specific antibody for target separation, thus

realize multi-functional roles in bioassay.

In the first system, we prepared dual-functional magnetic nanochains based on the facile polydopamine (PDA) strategy. In this study, the mixing and bioseparation behaviors of magnetic nanochains were thoroughly investigated by theoretical analysis and experimental observations. Our results demonstrated the magnetic nanochains immediately align in a magnetic field and undergo synchronous rotation in response to a rotating magnetic field at the maximum frequency of 540 rpm. In a microfluidic chamber, the mixing efficiency (σ_M) induced by magnetic nanochains can quickly reach to 80% within 1 min, making ideal nanomixer to promote fast fluid mixing and analyte transport in micro-scale assay devices. This, together with the readily functionalized surface, allows the magnetic nanochains to serve as both tiny stir bars for active liquid mixing and capture agents for targets of interest at high capture rate within 5 min.

Microarray techniques have found widespread uses in diverse fields ranging from fundamental genomic research to clinical diagnosis. However, static assays in conventional microarray platforms give rise to slow turnaround time due to diffusion-limited reaction kinetics. In the second system, we used PEGylated magnetic nanochains to improve DNA and Protein microarray assay. The rotating magnetic nanochains in sample solutions serve as tiny stir bars to promote dynamic mixing and effectively accelerate the transportation of targets to the

vicinity of the probes immobilized on glass surface. Our results have shown that the use of magnetic nanomixers led to improved kinetics, increased sensitivities, and reduced signal variation in both of DNA and protein microarray analysis. The magnetic dynamic nanomixers were expected to find widespread uses in on-surface bioassays that suffer from diffusion limitation, such as microfluidic assays, enzyme-linked immunosorbent assay (ELISA), and heterogeneous catalysis.

Finally, we used this dual-functional magnetic nanochains in microfluidic bioassay. It is well-recognized that while microfluidic biochips hold great promise in liquid analysis for biomedical research and clinical diagnosis. However, the devices of this size scale often present fundamental problems such as intrinsic poor liquid mixing and inefficient mass transfer across microfluidic channels and diffusion-limited on-surface assays, which create considerable obstacles to achieve rapid, ultrasensitive detection. In this study, we report a new type of magnetic nanochains integrated biochip (MiChip). In our MiChip assay, bioconjugated nanochains are actuated by tailored magnetic fields to play dual-functional roles as nanoscale stir bars and capture/enrichment agents, promoting concerted active liquid mixing and bioseparation. The use of dual-functional magnetic nanochains integrates the key functions of microfluidic bioanalysis (*i.e.*, liquid mixing, target bioseparation and signal transduction) into a streamlined process in a simple microfluidic system. In this study, we demonstrate that the

MiChip assay allows rapid, parallel analysis of small volumes ($\sim 1 \mu\text{l}$) of real clinical specimens. Cancer protein markers in serum samples from 20 cancer patients and specific bacteria in human saliva were sensitively and selectively quantified within 8 min.

Chapter 1 Introduction

1.1 Background

1.1.1 Diffusion Limitation in Surface-Based Bioassay

The identification and analysis of molecular and cellular biomarkers in body fluids represents a convenient and noninvasive way to gain insights into disease progression and therapeutic outcome^{1, 2}, creating a multitude of opportunities in medical diagnosis and prognosis of major human diseases such as cancer^{3, 4}, cardiovascular diseases^{5, 6}, infectious diseases⁷ and metabolic disorders⁸. In recent years, a great variety of bioassay techniques have been developed for rapid and accurate assay of a diverse range of biomarkers that undergo dynamic changes in body fluids. For example, enzyme-linked immunosorbent assay (ELISA)⁹, chemiluminescent immunoassay (CLIA)¹⁰, western blots¹¹, surface plasmon resonance (SPR) sensors¹², DNA/protein microarray¹³, and microfluidic biochip¹⁴ are among the most used analysis methods in clinical lab. Typically, all the above techniques are involved in the on-surface reaction. As shown in Figure 1.1, the DNA or protein capture probes were previously immobilized on the reaction surface and required to bind the targets (or reporter) in bulk solution during the analysis. In this regard, the delivery of targets to the vicinity of capture probes may play a crucial role in bioassay. Generally, the transport of molecules in solution is determined by the convective and diffusive effects. However, the

convective effect is usually limited owing to the spatial confinement in these bioassays, making the diffusion dominant in molecules transport. Hence, the assay kinetic of the surface-based assay can be either diffusion-controlled or binding rate-controlled determined by the surface binding kinetics and the diffusion rate. If the transport of analytes by the diffusion effects is much slower than the reaction that can bind them, the assay is diffusion-limited. On the contrary, the assay is binding rate-limited.

Figure 1.1 Surface based bioassay (protein and DNA microarray).

The average 1-D diffusion distance by a given molecule can be approximate by a simple equation¹⁵:

$$L \approx \sqrt{Dt} \quad (1)$$

where D is diffusion constant, L is the average diffusion distance, and t is the time it takes.

The diffusion constant (D) is defined by Stokes–Einstein equation¹⁶:

$$D = \frac{k_B T}{6\pi\eta r} \quad (2)$$

Where k_B is the Boltzmann constant, T is absolute temperature, η is the fluid viscosity, and the r is the hydrodynamic radius of target molecule. According to Eq 1 and Eq 2, the larger molecules have shorter diffusion distance, means they are harder to transport to the capture probe surface. In bioassay, the hydrodynamic radius of target molecules are usually very large, which leads the assay turnaround time very slow. This should be more remarkable in DNA assay due to the long length of DNA sequence. For example, the diffusion coefficient of a 250 bp DNA molecules is about $2 \times 10^{-7} \text{ cm}^2/\text{s}$, and thus, the typical diffusing distance of a 250 bp DNA is only 1.3 mm during an analysis time of 24 h.^{15, 17} The diffusion limitation of DNA makes the DNA microarray assay a long turnaround time of 12-24h, which limit its application in time-sensitive analysis.

Given the relatively rapid binding kinetics of DNA hybridization¹⁸ and antigen-antibody immune reaction¹⁹, a depletion layer next to the probe surface would be quickly generated, resulting in fundamental limitation in reaction time and sensitivity²⁰. Herein, it is highly desirable to develop an effective technique to accelerate the transport of targets in bulk solution. The magnetic stirring routinely used in macroscopic assays is natural to consider as a leading choice to promote fluids mixing in surface-based bioassay. However, owing to the spatial confinement in these assay system, no magnetic mixing system has been used in practice. Many methods such as acoustic waves agitation,²¹ electrokinetic delivery,²² cavitation microstreaming,¹⁷ air-driven bladders,²³ recirculating

flow,²⁴ and micromotor²⁵ have attempted to address the issue. However, these approaches necessitate additional devices that are not commonly used in biological and clinical labs, and inevitably increase the complexity of bioanalysis.

1.1.2 Mixing and Bioseparation in Microfluidic Device

Over the last decade, microfluidic technologies have experienced explosive growth, especially in medical diagnostics, due to the various advantages over conventional benchtop assays such as low samples and reagents consumption, short reaction time and high sensitivity.^{26, 27} The microfluidic-based techniques provide an ideal platform for miniaturized, portable, low-cost and non-invasive assay.^{28,29} Figure 1.2 shows a microfluidic platform for rapid genetic detection of *Mycobacterium tuberculosis* from patient body fluids in resource constrained environments.³⁰ All the components, such as PCR chamber, a 3-D serpentine mixing channel and micro NMR coils, are integrated into a miniaturized fluidic cartridge. The entire system is a disposable unit for *Mycobacterium tuberculosis* detection to prevent cross-talk.

Figure 1.2 A microfluidic platform for genetic detection of *Mycobacterium tuberculosis*. The PCR chambers, micro NMR coils and mixing channels are integrated on a chip for the streamlined assay.³⁰ Copyright 2013, Springer Nature.

In spite of the promising features, challenges remain in actual clinical application. For example, mixing of samples and reagents in microchannel is often very difficult because flow at the micro-scale typically is laminar and molecular diffusion across the microchannel is slow.³¹ Additional components should be introduced in microfluidic system for effective mixing (Mixing channels in Figure 1.2). In addition, biofunctionalized surface and complex interior structure are often introduced in microfluidic device to capture and separate the targets³², which both increase the difficulty of manufacturing. What is more, the separately developed mixing and bioseparation parts make the configuration of microfluidic device too complicate.

Mixing in microfluidic device

Microfluidics is the science and technology of manipulating very small volume of fluids, usually in the range of picoliters (10^{-12} L) to microliters (10^{-6} L), in networks of chambers and channels with dimensions from tens to hundreds micrometers.³³ The Reynolds number of flow in microfluidic channel with a width of 100 μm is the order of 0.1.³¹ Typically, the flow is laminar without turbulence when the Re number is smaller than 2300. Hence, the convective mixing cannot occur between two fluids in microfluidic channels, and the only mixing in microfluidic channels come from the molecules diffusion that is inherently very slow as discussed in Section 1.1.1.

To address this issue, a variety of micromixer designs have been exploited. Broadly speaking, they can be classified as either “passive” or “active”.^{34,35} Passive mixers rely on diffusion or chaotic advection, which generally demand complicated geometries of microchannels and lack the flexibilities.^{36, 25} Active mixers rely on external energy input like press, acoustics or electricity, enable fast and controllable mixing. However, the necessity to integrate external transducers (eg. pumps³⁷, or electrodes³⁸) generally complicates the whole system and limits its compatibility. Herein, it is necessary to develop an approach for fast and tunable mixing while not complicating the whole microfluidic system.

Bioseparation in microfluidic device

Although several assays have been realized on microfluidic system, there has been little success in commercialization of these technologies. Complex geometries^{39, 40} and biofunctionalized surfaces^{41, 42} have been introduced in microfluidic chip designs to perform various processes such as fluid mixing, pre-concentration of analytes and target detection. To increase the efficiency of target capturing, nano-sized structure⁴³ was introduced into the microchannel to increase the surface to volume ratio. These nanostructures are difficult or costly to be fabricated by standard industrial processes and prone to clogging. Figure 1.3 shows a microfluidic device containing nanostructured silicon substrate and chaotic micromixer for circulating tumor cells (CTC) capture.⁴⁴ In addition, sophisticated components for fluid handling, sample mixing, targets capturing and signal detection are developed separately and integration of these components into a single chip to perform actual clinical assay can be difficult. Herein, it is necessary to develop an approach to overcome these inherent limitations along with microfluidic assay.

Figure 1.3 Schematic representation of a microfluidic system containing nanostructured silicon substrate and chaotic micromixer for CTC capture.⁴⁴ Copyright 2013, John Wiley and Sons.

1.1.3 On-Chip detection based on SERS

Raman scattering is extremely inefficient in intrinsic, which is hard to be used for the directly detection of biomolecule with low abundance in bioassay.⁴⁵ Surface-enhanced Raman scattering (SERS) spectroscopy, using gold or silver nanostructure, have been widely exploited to address this issue.⁴⁶⁻⁴⁹ The plasmonic nanostructures with surface-confined electromagnetic field have been demonstrated to greatly amplify, by a factor of 10^6 or more, the intrinsically weak Raman scattering of molecules in close proximity.⁴⁵ The SERS based methods have been successfully applied for the analysis of extremely low concentration of analytes, even for single molecule detection.^{45, 50}

Anchoring Raman reporter molecules of different chemical structures on plasmonic nanostructures (Au or Ag nanoparticles) leads to an array of SERS-encoded nanoprobes with distinct, sharp spectroscopic signatures that can be

excited and differentially detected simultaneously, making them excellent multiplexing tags.^{51, 52} The sharp Raman peaks defined by molecular signatures makes it possible to develop a large library of nanoprobe by encoding identical plasmonic substrates with different Raman reporter molecules⁵² (Figure 1.4). Importantly, the SERS-encoded nanoprobe show better photostability than the commonly used fluorescent dyes.⁵³ Collectively, the SERS-encoded nanoprobe give rise to sensitive, stable, and spectrally-resolved signals with low background noise.

Figure 1.4 A library of SERS encoded nanoparticles with distinguishable SERS spectra.⁵⁴ Copyright 2006 American Chemical Society.

Furthermore, SERS spectroscopy only collects signal from a very small volume of sample, making it excellent compatibility with microfluidic devices. Figure 1.5 shows a schematic diagram of on-chip Raman spectroscopy.⁵⁵ The target area is first found out by the optical microscope. A laser beam is subsequently used to excite the sample. Finally, the Raman signal is collected by the spectrometer through dichroic mirror and notch filter.

Figure 1.5 Schematic illustration of a Raman spectroscopy integrated with a microfluidic unit.⁵⁵ Copyright 2013, Royal Society of Chemistry

In summary, the on-chip based Raman spectroscopy would benefit from its excellent compatibility with microfluidic devices⁵⁵, high sensitivity, multiplexed capacity and the superior photostability of Raman encoded probes.^{56, 57} Unlike the most used multiplexing systems, the SERS encoded method allows for simultaneous identification and quantification of multiple analytes in a single readout.

1.2 Motivation and Objectives

Microarray techniques have found widespread uses in diverse fields ranging from fundamental genomic research to clinical diagnosis. However, static assays in conventional microarray platforms give rise to slow turnaround time due to diffusion-limited reaction kinetics. Microfluidics that offer precise control of fluids and low sample and reagent consumption hold great promise in liquid analysis, which finds increasing uses in biomedical research and clinical diagnosis. In particular, there are growing demands for microfluidic biochips that allow for rapid, multiplexed detection of a panel of targets because of the pressing needs for dynamic profiling of analytes, timely diagnosis of heterogeneous diseases and high-throughput screening. However, the intrinsic poor liquid mixing and inefficient mass transfer in microfluidic channels and the diffusion-limited on-surface assays in current designs of microchips present a fundamental challenge to achieve rapid, ultrasensitive bioanalysis using simple microfluidic configurations. In this thesis, the overall objective is to develop an effective technique to address this long-standing challenge in microarray and microfluidic bioassay to make them more suitable for practical uses.

In view of the widespread use of magnetic stirring in macroscopic systems, we naturally consider to develop a micromixer which can be used in microscale device. Based on it, we synthesize magnetic nanochain with rigid structure by polydopamine strategy. The rigid polydopamine-coated magnetic nanochains

that we developed undergo synchronized rotation in a spinning magnetic field, analogous to macroscopic stir bars. To demonstrate the mixing performance of the magnetic nanochains, we apply them in microarray assay which usually suffer from serious diffusion limitation.

Importantly, polydopamine not only serves as the scaffold to lock the nanochain structure, but also allows for convenient surface functionalization by means of spontaneous Michael addition and/or Schiff base reaction with nucleophilic thiol and amine groups. As such, the magnetic nanochains endow with multifunction, such as mixing, bioseparation, and signal transduction. We attempt to utilize the bio-conjugated magnetic nanochains for sample mixing, targets recognition, separation, enrichment and detection in microfluidic chip. By integrating with the SERS detection, we propose to simultaneous identification and quantification of multiple biomarkers in a single readout.

Chapter 2 Review of the Literature

2.1 Polydopamine (PDA)

Polydopamine (PDA) have been extensively investigated after its discovery by Messersmith's group in 2007.⁵⁸ The invention of PDA was inspired by the adhesion phenomenon of mussel. Through the studies on the mussel adhesive proteins, the researcher found two key features (high catechol and high primary and secondary amine content) in *Mytilus edulis* foot protein (Mefp), especially in Mefp-3 and 5 may play a critical role in the adhesion (Figure 2.1). Based on this finding, they speculate the dopamine containing both amine and catechol functional groups may have similar adhesive properties like mussels. The further studies have demonstrated the PDA has excellent adhesive properties to virtually any organic and inorganic substrates.

Figure 2.1 (a) Photograph of a mussel attached to the PTFE. (b) Schematic of the location of Mefp-5. (c) Amine and catechol groups in Mefp-5. (d) The amino acid sequence of Mefp-5. (e) Molecular structure of dopamine. Copyright 2007, American Association for the Advancement of Science.

In the last decade, polydopamine (PDA) has emerged as a universal tool for surface coating due to its simplicity, low cost and versatile to virtually any solid materials.⁵⁹⁻⁶² In basic solution, the dopamine undergoes self-polymerization for spontaneous deposition of a PDA coating on the surface. The coating is conformal and is highly tailorable, making it suitable as a template for synthesis of nanostructure.⁶³⁻⁶⁶ The PDA coating further allows surface functionalization by means of spontaneous Michael addition and/or Schiff base reaction with nucleophilic thiol and amine groups.⁶⁷⁻⁶⁹

2.1.1 Adhesive Properties

The most outstanding properties of PDA is its strong adhesion on virtually any substrate. The PDA coating has been successfully formed on many types of materials, such as noble metal (Au, Ag, Pd, and Pt), metal oxide (Al_2O_3 , ZnO_2 , Fe_3O_4 , TiO_2), polymers (polystyrene, polyethylene, polypropylene, Polycarbonate, polyvinyl alcohol, polyacrylic acid, and poly(dimethylsiloxane)), SiO_2 , graphene, carbon nanotube, glass, diamond, claycalcium phosphate, GaAs, and QDs.^{58, 59, 70, 71} The catechol group in the structure of PDA is generally regarded as the crucial role in its strong versatility.^{58, 72} However, until now, the exact mechanism of PDA adhesion is not completely clear.

Generally, the adhesion interaction between PDA and the substrates can be classified into two types, namely, noncovalent binding and covalent binding.⁷² For example, in basic condition, PDA can covalently bind to the materials that containing thiol or amine group via Schiff base reactions and/or Michael addition.⁷³ The PDA also can form noncovalent binding with the substrate by π - π stacking, hydrogen bonding, metal coordination or chelating. For example, the PDA shows strong adhesion to metal oxide surfaces (Al_2O_3 , Fe_2O_3 , etc) via the coordination interaction between catechol group and the metal.⁷⁴

2.1.2 Versatile Reactivity

As shown in Figure 2.2, in weak basic condition (pH 8.5), the catechol groups in PDA were oxidized to quinone groups, which readily reacted with the nucleophilic amine groups or/and thiol groups through the Schiff base reaction or/and Michael addition reaction. The reactions between PDA and amine or thiol groups are simple and spontaneous without any coupling agent, which can offer a versatile platform for surface functionalization. Most of biomacromolecules (eg. DNA, RNA, protein, etc) contain amine and/or thiol groups. Hence, they can be easily functionalized to any types of materials through PDA coating. For example, trypsin, peptide and albumin have been successful modified on the cellulose paper, stainless steel and polyethylene membrane, respectively.^{69, 75, 76}

Figure 2.2 The reactions of polydopamine with thiol and amine groups *via* Michael addition and/or Schiff base reactions.

2.2 One-dimensional (1-D) Magnetic Nanostructures

Bioassay in microscale devices (eg. microarray and microfluidic chip) often suffers from the slow assay kinetics due to the diffusion limitation and intrinsic poor liquid mixing. In view of the widespread use of magnetic stirring in macroscopic systems, one would naturally consider it as a leading choice to promote liquid mixing in microscale devices. Magnetic nanoparticles have been widely applied in bioassays for performing multiple functions, such as labeling, capturing, separating and enriching analytes.^{77,78} Chain-like magnetic nanostructure, analogous to macroscopic stir bars, assembled from magnetic nanoparticles would naturally be considered as a leading choice to promote liquid mixing in microscale devices.

2.2.1 Transient Linked Magnetic Chains

Magnetic nanoparticles tend to form chain-like structures when placed in the uniform magnetic field due to the magnetic dipole-dipole interaction between the particles. The formed chains can be rotated by alternating magnetic fields, which can be developed to stir and mix fluids in a microenvironment.⁷⁹ Many studies, both experimental^{80, 81} and computational^{82, 83}, have investigated the dynamics of a magnetic particles chain by using the dimensionless Mason (Ma) numbers (ratio of viscous force to magnetic force). They found the magnetic nanochains quickly break up in a high Mason number that only mix the vicinity of magnetic nanochain. For a low Mason number, the magnetic nanochain can keep rigid and produce better mixing at the ends of magnetic nanochains. The best mixing located in an intermediate Mason numbers (a limited range), where the chain periodically fragments and reforms. However, the mixing behavior based on the break-reform are highly effected by the viscosity of fluids, which are not suitable for clinical application. What is more, the instability of the transient linked chains may lead them unstable in practical use. Hence, it is necessary to develop a method to permanent fix the magnetic chains.

2.2.2 Permanent Locked Magnetic Chains

A lot of materials, such as silica^{84,85} and polymers^{86,87,88} have been

developed to coat and permanently lock the magnetic nanochains. Chen *et al.* reported the using of silica to fix the aligned magnetic particles.⁸⁹ They prepared straight magnetic nanochains with tunable width from 75 nm to 1.4 μm and length with around 17 μm (Figure 2.3a). The as prepared magnetic nanochains were first used as nanomixer in a micro droplet. Yin *et al.* also fabricated the 1-D fixed nanochains by silica coating and studied its magnetically responsive photonic property (Figure 2.3b).⁸⁴ In the approaches, magnetic nanoparticles were permanently fixed in the chains and keep its structure after the field is switched off.

Other materials such as poly(cyclotriphosphazene-co-4,4'-sulfonyldiphenol) (PZS)⁸⁶, polyetherimide (PEI)⁹⁰, polystyrene (PS)⁹¹, and polymethacrylic acid (PMAA)⁹² were also successfully used to covalently link the aligned magnetic nanoparticles and fix the nanochains in the presence of the uniform magnetic field (Figure 2.3c and 2.3d). However, the polymers and silica based strategies are usually suffer from rigid reaction condition. In addition, the 1-D magnetic nanostructure prepared by the soft polymers are flexible, which are not suitable for liquid mixing. Moreover, the further surface functionalization on the polymers or silica coated magnetic nanochains are not easy or convenient to realize.

Figure 2.3 TEM images of magnetic nanochains prepared by different groups. (a) Nanometer-sized magnetic stir bars⁸⁹. (b) Magnetic nanochain@SiO₂⁸⁴. (c) Magnetic nanochain@poly(2-hydroxyethyl acrylate (HEA)-co-acrylic acid (AA))⁹³. (d) Magnetic nanochain@PZS⁸⁶. (a) Copyright 2013, John Wiley and Sons. (b) Copyright 2011, John Wiley and Sons. (c) Copyright 2017, American Chemical Society. (d) Copyright 2010, John Wiley and Sons.

2.3 Microarray

Microarray has been widely used in DNA, mRNA, protein and small molecules analysis.^{14, 94, 95} The global microarray market is expected to reach \$7.4 billion by 2018. Microarray allows for simultaneous detection of thousands of analytes in a single experiment, making it an ideal tool for high-throughput genomics and proteomics research.^{96, 97} For example, in a DNA microarray, thousands of

different oligonucleotide probes are immobilized on a solid surface at high spatial density, offering the possibility of obtaining a huge set of genomic data within a single experiment.⁹⁸

2.3.1 Microarray Assay and Its Challenges

DNA microarray assay

For DNA microarrays, Polymerase chain reaction (PCR) production sequence or synthesized oligonucleotides are printed on a solid substrate through covalent or non-covalent binding.^{99, 100} Each DNA spot contains picomoles of a specific DNA sequence, namely probes. Targets to be analyzed are fluorescently labeled and hybridized to their immobilized complementary capture probes (Figure 2.4).¹⁰¹ Microarray scanner was used to collect the emission characteristic of hybridized target. The emission information will be analyzed by a software to determine the relative abundance of gene sequences. Now, DNA microarrays built from more than 1,000 of different probes per square centimeter can generate large numbers of genomic data within a single test.¹⁰²⁻¹⁰⁴ Their use for the measurement of expression profiling and single nucleotide polymorphisms will have a big impact on disease diagnosis and biological research.¹⁰⁵⁻¹⁰⁸

Figure 2.4 Schematic illustration of cDNA microarray for expression profiling.¹⁰¹ Copyright 1999, Springer Nature.

Protein microarray assay

In recent years, the technique for high throughput analysis of thousands of different interested targets on a DNA microarray is now being applied in the field of proteomics.^{97, 109, 110} According to the recent publication of microarray assay, the protein microarray assay is becoming more popular. Many researchers are using the protein microarray to study the antigen-antibody reaction¹¹¹, DNA-protein reaction¹¹², enzyme-substrate¹¹³, and receptor-ligand assays¹¹⁴.

Challenges in microarray assay

Although the microarray method shows great power in high throughput DNA/protein detection, the majority of microarray assays experience the needs in improving assay speed and sensitivity due to the slow target diffusion from the bulk solution to surface-immobilized probes. Taking DNA microarrays as an example, the diffusion coefficient for target DNA in liquid solution is in the order of 10^{-6} to 10^{-7} cm^2/s . The scaling relationship $L = (DT)^{1/2}$ indicates that the target DNA can only moves 1-3 mm in 24 h due to static diffusion.²⁵ The DNA target diffusion rate is much lower than the target-probe binding kinetics, which creates a depletion layer near the complementary probe spot. As a result, the vast majority of target DNA in the liquid solution does not bind to its complementary probe, which often lead to low sensitivity.

2.3.2 Current Solutions to Improve Assay in Microarray

To overcome this diffusion barrier, many methods such as acoustic waves agitation,²¹ electrokinetic delivery,²² cavitation microstreaming,¹⁷ air-driven bladders,²³ drain-and-fill or recirculating flow,²⁴ and microfluidic chaotic mixer²⁵ were developed to accelerate liquid mixing and mass transfer.

As shown in Figure 2.5, Quake *et al.* integrated a complicated microfluidic device to circulate the sample solution between two microarray chambers.²⁵ They utilized herringbone indentations in the bridge channel promoted chaotic mixing. Compared with the static method, the signal increased 3-4 fold and the assay time was shorten to 2 h.

Figure 2.5 (a) Microfluidic Chaotic Mixing with two circulation chamber. (b) Microarray assay without mixing. (c) Microarray assay with chaotic mixing.²⁵ Copyright 2006, John Wiley and Sons.

Merkoçi *et al.* developed a self-propelled micromotor assisted microarray for protein detection (Figure 2.6).¹¹⁵ They found the micromotor assistance yielded a 3.5 fold higher signal intensity and reduced signal variation over static control. Other methods, such as cavitation microstreaming²¹ and electrokinetic methods²², also show signal improvement ranging from 2 to 5 fold.

Figure 2.6 Signal enhancement for protein microarray assisted by micromotors. Copyright 2014, John Wiley and Sons.¹¹⁵

2.4 Microfluidic Assay

Microfluidic systems offer precise control of fluids, low sample and reagent consumption, and short reaction time, which are highly desirable for the development of miniaturized, portable, and low-cost platforms for biomarker profiling in liquid samples^{26, 27, 116}. As shown in Figure 2.7, a microfluidic chip was fabricated by Quake's group to study the growth of microbial populations. This chip contains a high density of flow channels and pneumatic valves.¹¹⁷

Figure 2.7 A microfluidic device used to study the growth of microbial populations. This device includes a high density of pneumatic valves. Copyright 2005, The American Association for the Advancement of Science.¹¹⁷

Microfluidic assay chips have been designed for major human disease such as cancer^{118, 119}, cardiovascular diseases^{120, 121}, infectious diseases^{30, 122} and metabolic syndrome¹²³. Notably, two common structural components are often found in these chips: liquid mixer and biofunctionalized surface, although in different formats.^{124, 125} Spatial confinement in microchannels leads to low Reynolds number fluids that undergo laminar flow and have inefficient molecular diffusion across the channels³⁵. Passive or active mixtures are introduced to improve on-chip liquid mixing which is critical to molecular recognition of biomarkers¹²⁶⁻¹²⁸. Likewise, biofunctionalized surfaces are necessitated to separate and enrich biomarkers of interest from complex body fluids, which is key to specific biomarker detection in subsequent signal transduction.^{129, 130} Despite of the tremendous success in lab-scale demonstration of microfluidic liquid biopsy, these necessary built-in components inevitably increase structural, fabrication, and translational complexity of the chips, creating major barriers for practical medical applications.²⁷

2.4.1 Mixing in Microfluidic Device

At the micro-scale, fluid flows are laminar due to the low Reynolds number. As a consequence, microfluidic mixing is governed by molecular diffusion rather than turbulent mixing, and the speed of mixing is thus severely limited. To address these issues, a variety of micromixer designs have been exploited. Broadly speaking, they can be classified as either “passive” or “active”.^{34, 35}

Passive mixers rely on diffusion or chaotic advection, which generally demand complicated geometries of microchannels and lack the flexibilities (Figure 2.8a).³⁶ Active mixers rely on external energy input like press, acoustics or electricity, enable fast and controllable mixing. However, the necessity to integrate external transducers (eg. Pumps¹³¹, or electrodes³⁸) generally complicates the whole system and limits its compatibility (Figure 2.8b).¹³²

Figure 2.8 (a) Passive mixer based on serpentine channel with groove. (b) Active mixer based on bubble acoustic microstreaming. The piezo transducer is placed adjacent to the microfluidic device.¹³² Copyright 2009, Royal Society of Chemistry.

2.4.2 Bioseparation in Microfluidic Device

In microfluidic devices, biofunctionalized surface and complex interior structure are often introduced in microfluidic devices to capture and separate the targets of interest from complex fluid samples, which is key to specific detection in subsequent signal transduction. Fang *et al.* developed a microfluidic device (Figure 2.9) for capture and release non-small cell lung cancer (NSCLC) circulating tumor cells (CTCs).¹³⁰ In this device, single-stranded DNA-aptamers was grafted on the silicon nanowire substrates (SiNWS) to serve as a capture

agent. The nano-sized structure of SiNWS endow it with a higher capture efficiency. However, in practical, these nanostructures are difficult or costly to be fabricated by standard industrial process and prone to clogging.

Figure 2.9 (a) Image of a microfluidic device. (b) Cross-sectional view of the microchannel embedded in the aptamer-coated Chip. (c) Conceptual illustration of the molecular mechanism governing the capture and enzymatic release of NSCLC CTCs from the aptamer-coated SiNWS. Copyright 2013, John Wiley and Sons.

2.4.3 Integrated On-chip Liquid Mixing and Bioseparation

Despite recent success in the laboratory-scale demonstration of microfluidic bioanalysis, these necessary built-in components (micro mixer and Biofunctionalized surfaces) inevitably increase structural, fabricating, operational and translational complexity of the chips. It remains challenging to realize integrated liquid mixing, bioseparation and signal transduction in simple

microfluidic configurations.

Indeed, on-chip microfluidic mixing has been an active research area in the past 20 years, with numerous mixing strategies being proposed and demonstrated. However, most strategies in the literatures focus on serving the single function of mixing fluids at microscale. For example, acoustic mixing promotes microfluidic mixing through the transfer of vibrational energy from the ultrasonic sonotrode or piezoelectric element^{133, 134} to the mixing chamber. However, additional mechanisms such as magnetic field are necessarily appended to the setup to separate the target from the sample for subsequent detection.¹³⁵ The mechanisms for various on-chip functions (in addition to mixing function) generally introduce additional equipment and layers of complexity in the coupling of two or more different technologies. This poses a significant challenge in technology translation of microfluidics, from the perspective of cost, size, design and reliability of the product. As such, there is “a long-standing challenge” to have a simple and elegant microfluidic arrangement that not only can perform the mixing function, it can also serve other functions well in a compact, simple, reliable, and cost effective manner.

2.5 Surface-enhanced Raman Scattering (SERS)

Raman scattering is extremely inefficient in intrinsic, making it is not suitable for the direct application in the detection of biomolecule with low abundance.⁴⁵⁻⁴⁶

Plasmonic nanostructures are able to greatly amplify the intrinsically weak Raman scattering of molecules in close proximity via enhanced electromagnetic fields arising from excited localized surface plasmon resonance (LSPR). It is well-known that the Raman scattering of molecules adsorbed on the surface of metal nanoparticles (eg. Au or Ag nanoparticles) will be greatly amplified by an enhancement factor (EF) of 10^6 or more, making it possible for ultrasensitive detection.⁴⁷⁻⁴⁸ In certain cases, signal enhancement by 15 orders can be achieved. In 1997, single molecule SERS detection were separately reported by Nie⁴⁵ and Kneipp⁵⁰, where the Raman enhancement factors were estimated to be high as 10^{14} - 10^{15} . Furthermore, SERS spectroscopy allows for the integration with microscopic analysis techniques and is capable of collecting spectra from very small volumes (<1 uL), making it suitable for analysis on the microfluidics size scale.⁵⁵

2.5.1 SERS probe

The conventional SERS detection is the direct sensing of various analytes attached on the Au or Ag substrate, yielding both qualitative and quantitative information based on the SERS spectra of analytes. However, it is difficult to

apply in biodetection due to the low scattering cross-sections of biomolecules. Recently, SERS probes prepared by anchoring organic Raman reporter molecules on the plasmonic nanostructures (Au or Ag nanoparticles) and subsequently conjugated with recognition elements (*eg.* antibodies or aptamer) were widely used to indirectly detect the target biomolecules.^{51, 53} The SERS probes with distinct sharp, and strong Raman signatures that can be excited by single-wavelength laser, making them excellent for multiplexed detection. It is possible to develop a large library of SERS probes by encoding plasmonic nanostructures with different Raman reporter molecules.⁵⁴ The gold nanorods (AuNRs) are commonly used plasmonic substrates to prepare SERS encoded probes due to their easily tunable LSPR by changing the aspect ratio. It is known that matching the LSPR and the excitation laser wavelength is beneficial to SERS enhancement.

2.5.2 SERS Based Biodetection

The signal transduction based on SERS nanoprobe offers distinct advantages for multiplexed detection in unprocessed biofluids.⁵⁷ First, the sharp Raman peaks (full width at half maximum, 1-2 nm) defined by molecular signatures makes it possible to develop a library of nanoprobe.⁵⁴ These nanoprobe can be excited at a single wavelength and their SERS signals can be detected simultaneously for quantification of multiple targets.⁵² Second, the plasmonic nanostructures dramatically amplify the signal of Raman reporters to allow for ultrasensitive single-particle detection.^{45, 49} Raman enhancement by plasmonic substrates is

most efficient for molecules in 1-2 nm of close proximity because the electromagnetic field surrounding the substrates rapidly decays beyond that range.⁴⁸⁻⁴⁹ This effect and the intrinsically weak Raman scattering minimize the signal interference from external environment. Third, the scattering nature of the Raman signal imparts the nanoprobe with better photostability, compared with the commonly used fluorescent dyes.⁵⁶ Collectively, the SERS-encoded nanoprobe give rise to sensitive, stable, and spectrally-resolved signals with low background noise. In the event of detecting a panel of biomarkers, fingerprint signals of the nanoprobe allow for simultaneous identification and quantification of multiple biomarkers in a single readout, unlike the spatial encoding used in most multiplexing systems.

Chapter 3 Dual-functional Magnetic Nanochains:

Liquid Mixing and Bioseparation

3.1 Introduction

The identification and analysis of molecular and cellular biomarkers in body fluids represents a convenient and noninvasive way to gain insights into disease progression and therapeutic outcome, creating a multitude of opportunities in medical diagnosis and prognosis.^{1, 136, 137} In recent years, *in vitro* diagnosis (IVD) techniques such as enzyme-linked immunosorbent assay (ELISA)¹³⁸, DNA and protein microarray¹³⁹, microfluidic biochip¹⁴⁰, and surface plasmon resonance (SPR) sensors^{141, 142} have been greatly developed, driven by the demand for rapid, accurate and multiplexing detection of biomarkers. However, mixing is usually inefficient due to the small reaction volumes in these bioassay platform, which leads to slow turnaround and low sensitivity.

A large number of techniques have attempted to solve the mixing issue in bioassay. For example, many methods such as acoustic waves agitation²¹, electrokinetic delivery²², cavitation microstreaming¹⁷, air-driven bladders²³, drain-and-fill or recirculating flow²⁴, and microfluidic chaotic mixer²⁵ have been developed to accelerate the biochemical reaction in miniaturized devices. Although these methods allow shorter assay time and improved signal, they often suffer from complicated operation and low flexibility, and also increase structural

and fabricating complexity of the assay device. In view of the widespread use of magnetic stirring in macroscopic systems, one would naturally consider it as a leading choice to promote fluids mixing in microarray. However, owing to spatial confinement in these bioassays, no magnetic mixing system has previously demonstrated effective uses in practice.

Magnetic nanoparticles have been applied in microfluidic device for performing multiple functions, such as labeling, capturing, separating and enriching analytes.^{77,78} What is more, magnetic nanoparticles tend to form chain-like structures when placed in an uniform magnetic field due to the magnetic dipole interaction between the particles and the formed chains can be rotated by alternating magnetic fields, which can be developed to stir and mix fluids in a microenvironment.^{143, 144} Many studies, both experimental^{81, 145} and computational^{82, 83}, have investigated the dynamics of a magnetic particles chain, where the chain periodically fragments and reforms within a limited range of the Mason (Ma) numbers (ratio of viscous force to magnetic force). However, due to instability of the transient linked chains, the chains will break up when a certain maximum rotating speed is reached.¹⁴⁶ This make them inefficient in mixing and instable in clinical diagnosis application. To overcome this problem, silica^{84, 89} and various polymers^{86, 147, 148} were previously employed to coat and permanently lock the aligned magnetic particles. However, these coatings often suffer from multi-step synthesis or strict reaction condition and difficult to do further surface

functionalization.

Here, we reported a facile polydopamine (PDA) based strategy (Figure 3.1) to prepare dual-functional magnetic chains with rigid structure and readily reactive surface.^{149, 150} Dopamine undergoes self-polymerization at basic conditions to produce an adherent PDA layer, which strongly adhere on the aligned magnetic nanoparticles to permanently fix them. Importantly, PDA not only serves as the scaffold to lock the nanochain structure, but also allows for convenient surface functionalization by means of spontaneous Michael addition and/or Schiff base reaction with nucleophilic thiol and amine groups.⁶⁷ As such, the nanochains can be sequentially functionalized by target-specific capture antibody and thiolated poly(ethylene glycol) (PEG). In this chapter, the liquid mixing of magnetic nanochains in microscale device are numerically and experimentally studied. The bioseparation of cancer biomarkers by the magnetic nanochains is also investigated. We demonstrate the bioconjugated magnetic nanochains immediately align in a magnetic field and undergo synchronous rotation in response to a rotating magnetic field and show an excellent capture efficiency to 3 of cancer biomarkers.

Figure 3.1 Schematic illustration of the stepwise preparation of functionalized magnetic nanochains.

3.2 Experimental Section

3.2.1 Materials and Characterization

Iron(II) chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), Iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), dopamine hydrochloride, Bicine, sodium dodecyl sulfate (SDS), sodium chloride (NaCl), trisodium citrate, sodium acetate, and ethylene glycol were purchased from Sigma-Aldrich and used without purification. mPEG-SH (MW= 5000) were purchased from Laysan Bio. Su-8 100 was purchased from MicroChem. Sylgard 184 silicone elastomer kit was obtained from Dow Corning. Prostate specific antigen (PSA), carcinoembryonic antigen (CEA) and α -fetoprotein (AFP) were obtained from Fitzgerald. Capture antibodies to PSA were purchased from Genscript. Capture antibodies to AFP and CEA were obtained from Meridian Life Science. Ultrapure water ($18.2 \text{ M}\Omega \cdot \text{cm}$) was purified by Milli-Q ultrapure water systems and used in all experiments. Scanning electron microscopy (SEM) image was captured using a field-emission scanning electron microscope (JSM-6700F) with an acceleration potential of 10 kV. Transmission electron microscopy (TEM) image was obtained on a Jeol JEM 2010 electron microscope at an acceleration voltage of 300 kV.

3.2.2 Synthesis of Magnetic Nanoparticles

The magnetic nanoparticles were synthesized by a modified solvothermal reaction.¹⁵¹ Briefly, FeCl_3 (0.54 g) and trisodium citrate (0.20 g) were first

dissolved in ethylene glycol (20 ml), and sodium acetate (1.20 g) was added afterwards under stirring. The mixture was stirred vigorously for 30 min and then sealed in a Teflon-lined stainless-steel autoclave (30 ml capacity). The autoclave was heated at 200 °C and maintained for 10 h, and then allowed to cool to room temperature. The black products were washed 5 times with ethanol and deionized (DI) H₂O. The size of magnetic nanoparticles can be controlled by changing the initial concentration of FeCl₃.

3.2.3 Synthesis of Magnetic Nanochains

To prepare the magnetic nanochains, 1 mg of magnetic nanoparticles were dispersed in 18 ml of bicine buffer (pH 8.5, 10 mM), followed by adding 8 mg dopamine. The mixture was immediately placed in a uniform magnetic field (0.05 T) for 15 min and then left undisturbed for 12 h. After the incubation, the product was separated with a permanent magnet and washed with DI H₂O for 3 times. The final product was dispersed in 1 ml of DI H₂O and stored at 4 °C before use. The length and width of the resulting magnetic nanochains were controlled by changing the magnetic reaction time and the initial dopamine concentration.

3.2.4 Preparation of Antibody-Conjugated Magnetic Nanochains

The harvested nanochains were washed 3 times with DI H₂O and dispersed in 5 ml bicine buffer (pH 8.5, 10 mM). 100 µl antibody solution (Ab-I, 0.25 mg ml⁻¹) was added to the dispersion and the mixture was shaken for 12 h at room

temperature. Afterwards, 1 ml mPEG-SH solution (10 mg ml^{-1}) was added to the mixture to block the unreacted sites for 12 h. The excess reactants were removed by repeated magnetic separation and washing. Finally, the bioconjugated magnetic nanochains were resuspended in 1 ml PBS (0.01 M, pH 7.4) and stored at 4 °C before use.

3.2.5 Fabrication of Y-Shape Microfluidic Chip

The Y-shape microfluidic chip was fabricated by stacking two patterned PDMS layers on a glass slide.¹⁵² The bottom PDMS layer contains the Y-shape fluid channels and a mixing chamber while the top layer hosts the pneumatic control channels. Protruding mold patterns for these channels were fabricated via conventional photolithography on silicon wafers with SU-8 50 photoresist. PDMS and curing agent were mixed in ratios of 20:1 and 5:1 (w/w) and degassed in a vacuum chamber. The 20:1 PDMS mixture was spun on the fluid channel mold at 1500 rpm for 60 s while the 5:1 PDMS mixture was poured on the control valve mould. Both molds were then baked at 80 °C for 30 min. Access holes were made for pressure control with a hole puncher and the control valve layer was aligned to the fluid channel layer using a customized alignment tool. The two layers were bonded together by baking the assembly at 80 °C for 2 h. After punching the inlet/outlet holes for fluid access, the chip was bonded to a glass slide through the oxygen plasma treatment. The surfaces of the channels are modified to be hydrophilic by coating of PDA and PEG-SH to suppress potential

biofouling.

3.2.6 Electromagnetic Coils Setup

The working principle of the electromagnets providing a rotating magnetic field is similar to a two phase brushless DC motor. The four coils are mounted on a cross-shaped aluminum plate. Each coil consists of windings maximizing the space allowed by a quadrant on the plate. Ferrite cores were inserted in the coils to increase the magnetic flux. A function generator supplies the reference pulse trains for a stepper motor driver to generate the two phases of high current excitation at a microstepping setting of 1/128 to approximate a sinusoidal waveform. The coils generate a rotating magnetic field with adjustable frequencies in the range of 1 - 11 Hz (60 - 660 revolutions per minute).

3.2.7 Mixing Evaluation

The efficiency of fluid mixing by the rotating magnetic nanochains were evaluated in a simple Y-shaped microfluidic chip, in which two channels of fluids stream into a circular mixing chamber (300 μm in diameter) that can be closed by two pneumatic microvalves. Magnetic nanochains were injected via two separate inlets into the mixing chamber at a flow rate of 50 $\mu\text{l h}^{-1}$, with one of the fluids highlighted by CdSe/ZnS quantum dots (QDs) with emission centered at 550 nm. Subsequently, the valves were closed and the alternating magnetic field was applied to actuate the magnetic nanochains to mix the two fluids. The QDs

serve as a fluorescent marker to quantify the mixing efficiency (σ_M) between the two streams by tracking the fluorescence distribution in the closed mixing chamber.

3.2.8 Evaluation of Target Capturing Efficiency

Antibody-conjugated magnetic nanochains were mixed with 1 ml of relevant biomarkers spiked samples in the centre of electromagnetic coils for 1-15 min. Subsequently, the magnetic nanochain-Ab-target immune complexes were separated by a permanent magnet for 3 min. The capture efficiency was defined as the ratio of the targets captured by the nanochains to the total amount of targets originally present. The amount of captured targets was calculated by the total amount of targets minus unbound targets in solution, which was measured by the commercial ELISA kits.

3.3 Results and Discussion

3.3.1 Synthesis of Magnetic Nanoparticles

Highly water-dispersible magnetite particles with uniform size were synthesized with citrate as the stabilizer through a modified solvothermal approach. SEM images (Figure 3.2) show that the average diameter of the magnetic nanoparticles increases from 80 to 350 nm with the increasing initial concentration of FeCl_3 . Here, we choose the magnetic particles with average diameter of 250 nm (Figure 3.2d) for all the next experiments.

Figure 3.2 SEM images of Fe_3O_4 particles synthesized with different initial FeCl_3 concentrations: (a) 0.05; (b) 0.1; (c) 0.15; (d) 0.2; (e) 0.25; (f) 0.3 mol/L. Scale bars: 200 nm.

3.3.2 Synthesis of Magnetic Nanochains

To prepare magnetic nanochains, superparamagnetic Fe_3O_4 nanoparticles of 250 nm diameter (Figure 3.2d) dispersed in dopamine solution of bicine buffer (pH 8.5, 0.1 mM) are placed in a homogeneous magnetic field (0.05 T) to form linear arrays. Self-polymerization of dopamine at a weak basic condition leads to highly crosslinked, rigid polydopamine that shows strong adhesion against almost any solid substrate.⁵⁸ As a result, the adhesive polydopamine forms a conformal coating on the aligned nanoparticles, leading to crosslinked magnetic nanochains (Figure 3.3).

Figure 3.3 SEM (a) and TEM (b) image of the magnetic nanochains

We further investigate the hysteresis loops of as-synthesized magnetic nanoparticles and magnetic nanochains (Figure 3.4). The results show the magnetic nanoparticles and magnetic nanochains are both superparamagnetic. Magnetization saturation values (M_s) of the magnetic nanoparticles and magnetic chains are 59.7 and 41.7 emu/g, respectively. The lower magnetization of

magnetic nanochains is because a layer of non-magnetic polydopamine is deposited on the nanochain during its formation.

Figure 3.4 Hysteresis loops of as-synthesized magnetic nanoparticles and magnetic nanochains.

3.3.3 Length and Width of Magnetic Nanochains

The length and width of the resulting magnetic nanochains were controlled by changing the magnetic reaction time and the initial dopamine concentration (Figure 3.5). The average length of the nanochains grew from about 10 μm (9.8 ± 1.4 , $n=10$) to 20 μm (20.3 ± 2.2 , $n=10$) when the reaction time in the magnetic field was extended from 10 to 30 min. The nanochains formed at this stage were fragile and easily broke into shorter fragments of about 3 μm (3.3 ± 0.5 , $n=10$) after 3 s of ultrasonication, allowing for preparation of shorter nanochains. Subsequent reaction in absence of the magnetic field led to permanently fixed nanochains by continuous deposition of polydopamine. The width of the

nanochains is primarily dependent on the initial concentration of dopamine, with concentrations of 0.2, 0.45, and 0.9 mg ml⁻¹ leading to nanochain widths of about 300 (297 ± 18 , $n=10$), 400 (398 ± 30 , $n=10$) and 600 (589 ± 43 , $n=10$) nm, respectively.

Figure 3.5 SEM images of magnetic nanochains of different length and width. (a – c) 20, 10, and 3 μm in length, respectively. (d – f) 300, 400, and 600 nm in width, respectively.

3.3.4 Theoretical Analysis for the Rotation of Magnetic Nanochains

The magnetic chains immediately align in a magnetic field and undergo synchronous rotation in response to a rotating magnetic field, offering the possibility of using them as nanoscale stir bars.^{82, 153} When a rotating magnetic field is applied, two opposing angular torques (*i.e.*, magnetic and viscous torque) act on the magnetic nanochains (Figure 3.6). For the nanochains to maintain a constant angular velocity, the opposing torques need to be balanced at the center

of the chain.¹⁵⁴ The maximum frequency ($f_{\max}$) of the applied alternating field for synchronous rotation of the nanochains is deduced as follows.¹⁵⁴

Figure 3.6 Force analysis of a magnetic nanochain in a rotating external magnetic field. r_0 and r_c are the radius of magnetic nanoparticles and the thickness of polydopamine (PDA) layer, respectively.

When an external magnetic field with magnitude H_0 is applied, the nanoparticles acquire an induced dipole moment μ :

$$\mu = \frac{4}{3} \pi \mu_0 \chi H_0 r_0^3 \quad (1)$$

where r_0 is the radius of the particle, μ_0 is the permeability constant and χ is the magnetic susceptibility. To simplify the analysis, the effect of induced magnetic field of the paramagnetic particles on their neighbouring particles is neglected. The magnetic torque acting on a chain of N units of such spherical particle is given by^{155, 156}:

$$\Gamma_m = \frac{3\mu^2 N^2}{64\pi\mu_0 r_0^3} \sin(2\alpha) \quad (2)$$

where α is the angle between the applied magnetic field and the longitudinal direction of the chain. The viscous torque which opposes the rotation of the chain

is given by:

$$\Gamma_v = \kappa V \eta \omega \quad (3)$$

where η is the viscosity of the fluid, ω is the angular frequency of the rotating magnetic field, V is the volume of the magnetic chain, and κ is the shape factor.

The volume of the entire magnetic chain can be approximated as:

$$V = N \frac{4\pi(r_0 + r_c)^3}{3} \quad (4)$$

where r_c is the thickness of the PDA coating which surrounds the magnetic nanoparticles to form the chain. The shape factor of a linear rigid chain formed by N spherical beads is given by

$$\kappa = \frac{2N^2}{\ln(N/2)} \quad (5)$$

A constant angular velocity is achieved when the two opposing angular torques balance each other at the center of the chain. The maximum value for α to maintain synchronous rotation between the chain and the applied magnetic field is $\pi/4$. Hence, the maximum applied frequency for synchronous rotation, f_{max} can be found by equating Eq 2 to Eq 3:

$$f_{max} = \frac{\chi^2 H_0^2 \ln(N/2)}{16\pi N \eta \mu_0} \left(\frac{r_0}{r_0 + r_c} \right)^3 \quad (6)$$

In this experiment, the magnetic nanochain length (L) is approximated as $N \times 2r_0$.

Hence, Eq. 6 can be substituted as:

$$f_{max} = \frac{\chi^2 H_0^2 r_0 \ln(L/4r_0)}{8\pi L \eta \mu_0} \left(\frac{r_0}{r_0 + r_c} \right)^3 \quad (7)$$

In the Eq. 7, the terms of π ($= 3.14$) and μ_0 ($= 1.26 \times 10^{-6} \text{ H m}^{-1}$) are constant. The

strength of applied magnetic field (H_0) and the magnetic susceptibility (χ) are measured to about 633.70 A m^{-1} and 9.04 , respectively. The viscosity of the fluid (η) is assumed to be the same as that of water ($8.90 \times 10^{-4} \text{ Pa.s}$). The radius of the magnetic nanoparticles (r_0) is fixed at 125 nm in this study. Hence, based on Eq. 7, it can be determined that $f_{\max}$ increases with decreasing chain length (L) and thickness of the PDA coating (r_c) (Figure 3.7). This means that higher rotation speed can be achieved with shorter chain and thinner coating. On the contrary, longer and thicker chains displace more liquid as they rotate and generate more pronounced convection to promote mixing. Hence, the chain length and thickness need to be optimized to achieve the highest mixing efficiency by seeking a balance between the chain size and rotation speed.

Figure 3.7 Theoretical analysis results of maximum frequency ($f_{\max}$) to maintain synchronous rotation of the magnetic nanochains with different length (a) and PDA thickness (b).

3.3.5 Experimental Observation of the Rotation of Magnetic Nanochains

Dark-field imaging (Figure 3.8) shows the magnetic nanochains immediately aligned when placed in a homogeneous magnetic field (0.008T), and took synchronous, localized rotation when two pairs of orthogonal electromagnetic coils were activated periodically.

Figure 3.8 Dark-field microscope images of magnetic nanochains responding to an alternating magnetic field, captured by a CCD camera.

The time-lapse high-speed camera observation (Figure 3.9) shows that the nanochains (length: 20 μm , width: 400 nm) can achieve a maximum synchronous rotation speed of 9 Hz (540 rpm) and start to exhibit a small degree of hysteresis at an applied magnetic field of 11 Hz, which is consistent with the speed (10.4 Hz or 624 rpm) estimated in theoretical analysis. Beyond that, the driving magnetic torque generated by the field is insufficient to balance the opposing viscous drag, which increases linearly with the angular velocity.

Figure 3.9 Rotation frequency of magnetic nanochains observed by time-lapse high-speed camera. The rotating magnetic nanochains (20 μm in length and 400 nm in width) in the alternating magnetic field (Frequency ranged from 1-11 Hz) were imaged by a high speed camera (500 frames per second).

3.3.6 Experimental Setup for Evaluation of Mixing Efficiency by Magnetic Nanochains

We evaluated the efficiency of fluid mixing by the rotating magnetic nanochains in a simple Y-shaped microfluidic chip, in which two channels of fluids stream into a circular mixing chamber (300 μm in diameter) that can be closed by two pneumatic microvalves (Figure 3.10a). Magnetic nanochains were injected via two separate inlets into the mixing chamber with one of the fluids highlighted by CdSe/ZnS quantum dots (QDs). The device was placed in the centre of electromagnetic coils mounted on the stage of fluorescence inverted microscope. (Figure 3.10b)

Figure 3.10 (a) Y-shape microfluidic chip for the evaluation of mixing efficiency. (b) Picture of experimental setup for evaluation of mixing efficiency

The QDs serve as a fluorescent marker to quantify the mixing efficiency (σ_M) between the two streams by tracking the fluorescence distribution in the closed mixing chamber. The mixing efficiency between the two streams of fluid can be quantified by examining the captured fluorescent images. A MATLAB program was written to evaluate the mixing efficiency based on the changes of the fluorescent images when the two fluid streams mix. The mixing efficiency, σ_M can be defined as:

$$\sigma_M = \left[1 - \frac{\sqrt{\frac{1}{N} \sum_{i=1}^N (\bar{I}_i - \bar{I}_\infty)^2}}{\sqrt{\frac{1}{N} \sum_{i=1}^N (\bar{I}_{0i} - \bar{I}_\infty)^2}} \right] \times 100\% \quad (8)$$

where N is the total number of points examined in the cross-stream direction, $\bar{I}_i$ is the normalized intensity at each point, $\bar{I}_{0i}$ is the normalized intensity at each point with no mixing taking place (value 0 or 1), and $\bar{I}_\infty$ is the normalized intensity in the complete mixing states (value 0.5). Mixing efficiencies between the two fluids were calculated based on Eq 8, where $\sigma_M = 0$ indicates no mixing

occurs, while $\sigma_M = 100\%$ represents complete mixing. Quantitatively, σ_M of 0 and 100% represents no mixing and a complete mixing, respectively.

3.3.7 Elimination of the Black Magnetic Chains in Image

As shown in Figure 3.11a, the black magnetic chains in the image interfere on the evaluation of fluorescence intensity distribution. To address the problem, a permanent magnet was placed above the chamber to align the magnetic chains in the perpendicular direction so that the black magnetic chains became invisible from sight to enable accurate evaluation of the mixing efficiency (Figure 3.11b). Figure 3.11c indicates that the intensity profiles before and after the deployment of the magnet do not differ significantly. Moreover, by hiding the magnetic chains, the intensity profile is much smoother and illustrates a more accurate representation of the mixing condition in the chamber.

Figure 3.11 Elimination of the impact of magnetic chain images in the evaluation of mixing efficiency. (a) Image of mixing chamber before placing a magnet. (b) Image of mixing chamber after placing a magnet. (c) Fluorescent intensity profiles evaluated across the centre of the mixing chamber (white arrows).

3.3.7 Mixing Enhancement by Magnetic Nanochains

Time-lapse observation shows that σ_M is dependent on both structural parameters (length and width) and concentration of the nanochains (Figure 3.12). For example, in a magnetic field with an alternating frequency of 5 Hz, nanochains with an average length of 20 μm and 3 μm led to σ_M of 81% and 35% respectively after 1 min of mixing. Notably, although σ_M improves with increasing chain width and concentration, it shows marginal improvements after the width and concentration reach 400 nm and 0.25 mg ml^{-1} respectively. Therefore, magnetic chains of 20 μm in length and 400 nm in width at a concentration of 0.25 mg ml^{-1} was eventually chosen for optimized mixing.

Figure 3.12 Mixing enhancement by using magnetic chains of different lengths (a), widths (b) and concentrations (c).

We also investigated the rotational speed on mixing efficiency. The mixing efficiency of magnetic nanochains and magnetic nanoparticles at different magnetic fields was evaluated in 1 min. The results show the mixing efficiency of magnetic nanochains increases with increasing spinning frequency and level off at 5 Hz (300 rpm) (Figure 3.13). In clear contrast, magnetic nanoparticles of the same concentration only led to 21% of mixing, similar to the same sample in absence of the spinning magnetic field (Figure 3.12), underlining the importance of liquid mixing by the nanoscale stir bars.

Figure 3.13 Mixing efficiency of magnetic nanochains and magnetic nanoparticles in spinning magnetic fields of different frequency.

3.3.8 Targets Capture Efficiency by Magnetic Nanochains

Our magnetic nanochains not only act as rigid nanoscale stir bars to enhance fluid mixing, but also function as target-specific capture agents to capture

analytes. We investigated the capture rates by magnetic nanochains from 1 to 15 min. The results show the magnetic nanochains can capture more than 80% of PSA (100 ng ml^{-1}) for 5 min of mixing (Figure 3.14). We reason the high capture efficiency was produced from the continuous rotating of magnetic nanochains that accelerate the transport of targets to the vicinity of capture antibodies on the chains.

Figure 3.14 Capture rate of PSA versus mixing time. PSA (100 ng ml^{-1}) was mixed and reacted with anti-PSA magnetic nanochains. The capture rates were determined by ELISA.

A mixture of cancer biomarkers (PSA, CEA and AFP) were incubated with magnetic nanochains that functionalized with either anti-PSA, anti-CEA, anti-AFP or a mixture of the three antibodies. The achieved capture efficiency was determined using ELISA upon magnetic separation (Figure 3.15). The magnetic chains show high capture efficiency (90-95%) against the relevant targets with low crosstalk against the mismatched targets (< 5%). The multiplexed nanochains can simultaneously capture the three targets with a slight drop of capture

efficiency (85-88%).

Figure 3.15 Capture efficiency of cancer biomarkers by magnetic nanochains for 15 min of mixing.

3.4 Conclusion

Liquid mixing has been a long-standing challenge in bioassays due to the slow molecular diffusion and the intrinsic laminar flow in spatially confined fluids.^{157, 158} In this study, we developed a polydopamine-based strategy to produce robust magnetic nanochains with high loading of magnetic components, which leads to easy, reproducible manipulation of the nanochains by tailored magnetic fields. Our theoretical analysis and experimental observations have demonstrated the magnetic nanochains immediately align in a magnetic field and undergo synchronous rotation in response to a rotating magnetic field at the maximum frequency ($f_{\max}$) of 9 Hz or 540 rpm. In a microfluidic chamber, the mixing efficiency induced by magnetic nanochains can quickly reach to 80% within 1 min, making them ideal nanomixer to promote fast fluid mixing and

analyte transport in micro-scale assay devices. This, together with the readily functionalized surface, allows the magnetic nanochains to serve as both tiny stir bars for efficient liquid mixing and capture agents for targets of interest at high capture efficiency ($CE > 80\%$ for 5 min of mixing).

3.5 Declaration

This work presented in this chapter has been published in *Nature Communications*.

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Chapter 4 Dynamic Magnetic Nanomixers for Improved Microarray Assays by Eliminating Diffusion Limitation

4.1 Introduction

Microarray techniques have found widespread uses in diverse fields ranging from fundamental genomic research to clinical diagnosis.¹⁵⁹⁻¹⁶¹ Microarray allows for simultaneous detection of thousands of analytes in a single experiment, making it an ideal tool for high-throughput genomics and proteomics research.^{97, 105, 162} For example, in a DNA microarray, thousands of different oligonucleotide probes are immobilized on a solid surface at a high spatial density, offering the possibility of obtaining a huge set of genomic data within a single experiment.^{101, 162} However, microarray assays face a common challenge in slow turnaround and low sensitivity due to the slow molecular diffusion from bulk solutions to surfaces with immobilized probes.^{15, 20, 115} For example, without mixing, the typical diffusing distance of a 250 bp DNA with a diffusion constant of $\sim 2 \times 10^{-7}$ cm²/s is only 1.3 mm during an analysis time of 24 h.^{15, 17} As a result, the vast majority of DNA target molecules in bulk solution have no access to the complementary DNA probes (Figure 4.1a). If target molecules are in low-abundance in solution, a depletion layer next to the probe surface would be quickly generated upon targeting binding to the substrate, resulting in fundamental limitation in reaction time, sensitivity and repeatability.^{20, 96} Herein,

it is highly desirable to develop an effective method to continuously mix the sample solution at low Reynolds number and deliver targets rapidly to the vicinity of the surface-anchored probes.

In our previously work, we have demonstrated fast and high-efficient mixing in microfluidic chamber by using magnetic nanochains as dynamic nanomixers. Here, we report a dynamic magnetic nanomixer by using PEGylated magnetic nanochains as nanoscale stir bars.^{149, 150} The magnetic nanochains immediately align in a magnetic field and undergo synchronous rotation in response to a rotating magnetic field, making them ideal nanomixer to promote fast fluid mixing and analyte transport in microarrays.

In microarray analysis, the magnetic nanochains are premixed with liquid samples and loaded in the microarray chamber. The rotating magnetic nanochains in sample solutions serve as tiny stir bars to promote dynamic mixing and effectively accelerate the transportation of target molecules to the vicinity of the complementary probes than that incurred by static diffusion alone, as illustrated in Figure 4.1b. The rapid mixing minimizes the localized target depletion, resulting in rapid turnaround, improved sensitivity and reduced spot-to-spot variation.^{15, 25, 115} Unlike commercial mixing solutions that are currently adopted in microarrays, our magnetic nanochains based approach doesn't require any extra built-in components, neither does it increase the structural, fabrication, and

operational complexity of the microarray. The magnetic nanochain based assay is fast, flexible, economical, simple to operate, and compatible with any commercial microarrays.

Figure 4.1 Schematic illustration of the magnetic nanomixer in microarray. (a) Conventional hybridization via static diffusion. (b) Eliminate diffusion limitation by using magnetic nanochains as dynamic nanomixer.

4.2 Experimental Section

4.2.1 Materials

Iron(II) chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), Iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), dopamine hydrochloride, Bicine, sodium dodecyl sulfate (SDS), sodium chloride (NaCl), trisodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$), HEPES, and BSA were purchased from Sigma-Aldrich and used without purification. mPEG-SH (MW= 5000) were purchased from Laysan Bio. *E. coli* O157:H7 ssDNA and fluorescence labeled DNA probe were order from Sangon Biotechnology. PSA standard protein was purchased from Fitzgerald. Capture and detection antibody to PSA were obtained from GenScript. Ultrapure water ($18.2 \text{ M}\Omega \cdot \text{cm}$) was purified by Milli-Q ultrapure water systems and used in all experiments.

4.2.2 Preparation of PEGylated Magnetic Nanochains

The magnetic nanoparticles with average diameter of 200 nm were synthesized by following the method in Section 3.2.2. Then, 1 mg of the magnetic nanoparticles were dispersed in 18 ml of Bicine buffer (pH 8.5) and mixed with 8 mg of dopamine. The mixture was immediately placed in a uniform magnetic field (0.05 T) for 15 min and then left undisturbed for 12 h. In absence of the magnetic field, the dopamine undergoes polymerization to continuously deposit on the aligned magnetic nanochains to permanently fix them. After the incubation, the product was separated with a permanent magnet and dispersed in 1 ml of DI H_2O . The harvested nanochains were then washed 3 times with DI H_2O and

dispersed in 5 ml of Bicine buffer (pH 8.5). 1 ml of mPEG-SH solution (5 mg ml⁻¹) was added to the dispersion and the mixture was shaken for 12 h at room temperature. The excess reactants were removed by repeated magnetic separation and washing. Finally, the PEGylated magnetic nanochains were resuspended in 1 ml of PBS (0.01 M, pH 7.4) and stored at 4 °C before use.

4.2.3 Characterization of Magnetic Nanomixers

Scanning electron microscopy (SEM) image was captured using a field-emission scanning electron microscope (JSM-6700F) with an acceleration potential of 10 kV. Transmission electron microscopy (TEM) image was obtained on a Jeol JEM 2010 electron microscope at an acceleration voltage of 300 kV. Two-dimensional (2D) numerical simulation of magnetic nanomixers was implemented on COMSOL Multiphysics software.

4.2.4 Fabrication of DNA and Protein Microarray Slides

NH₂ modified DNA probes or capture antibodies was suspended in spotting buffer (3×SSC) at concentration of 100 μM or 2.5 mg ml⁻¹. The DNA probes and capture antibodies were spotted on the epoxy coated glass slides (SuperEpoxy, Arrayit co.) using μArrayer™ (LabNEXT) with a pin diameter of 140 μm. After printing, the slides were incubated at 37°C overnight in a low humidity environment. The spotted slides were assembled with ProPlate frame (16 wells, Grace Bio-Labs) to create separate reaction wells (7 × 7 × 7 mm). Each well was

blocked with 200 μ l blocking buffer (0.1% BSA in 5 $\times$ SSC) at room temperature for 3 h and then washed with 200 μ l 1 $\times$ washing buffer (0.1% SDS in 5 $\times$ SSC) for 3 times.

4.2.5 Microarray Assay

The *E. coli* O157:H7 target DNA¹⁶³ and PSA standard were diluted with hybridization solution (1 $\times$ TAE-Mg or 1 $\times$ PBS) into a series of solutions at desired concentration. Samples were then separated into aliquots and added in the microarray reaction wells, which was placed in the custom magnetic stirrer. Magnetic nanochains were added into a group of solutions and actuated by the rotating magnetic field for dynamic mixing, while the other group were performed as static control. After reaction at 42 $^{\circ}$ C for 1 h (or other specified time), the wells were washed with 1 $\times$ washing buffer for two times and then 0.1 $\times$ washing buffer for two times. The slides were disassembled from the frame and immediately placed in a 50 ml centrifuge tube to dry them by centrifuging for 5 min at 2000 rpm. A total internal reflection (TIRF) microscopy (ECLIPSE Ti2, Nikon) was used to detect the signals of microarray. All fluorescence images were acquired at 500 ms exposure and then analyzed by software MetaVue. The final signal intensities were the intensities of spots signal subtracted by the intensities of local background signal.

4.3 Results and Discussion

4.3.1 PEGylated Magnetic Nanochains

We previously developed a polydopamine-based approach to preparing magnetic nanochains with rigid structure and PEGylated surface.^{149, 150} The TEM image (Figure 4.2) demonstrates the conformal coating of the PDA adhesive on the aligned magnetic nanoparticles. PDA not only serves as the adhesive to lock the nanochain structure, but also allows for straightforward PEGylation to minimize biofouling via spontaneous Michael addition and/or Schiff base reaction with nucleophilic thiolated PEG (PEG-SH) groups.¹⁶⁴ The length and width of the magnetic nanochains can be controlled by the reaction time and the initial dopamine concentration.¹⁴⁹ PEGylated magnetic nanochains with an average length of 20 μm and a width of 400 nm (Figure 4.2) were selected for all experiments in microarray.

Figure 4.2 SEM image of PEGylated magnetic nanochains. Inset: TEM image of magnetic nanochains.

4.3.2 Numerical Simulation of Liquid Mixing by Magnetic Nanochains

Magnetic nanochains align and undergo synchronous rotation in response to a rotating magnetic field, offering the possibility of using them as dynamic nanomixers. Two-dimensional (2-D) numerical simulation is performed to investigate the liquid mixing in Newtonian fluids.^{82, 83} The simulation consists of a stationary outer boundary (75 μm length and 50 μm width), and two rotating parts representing two magnetic nanochains (each with 20 μm length and 400 nm width). The top and bottom halves of the simulation domain are filled with fluid A (red) and fluid B (blue), respectively. Once a magnetic field is applied, the rotating magnetic nanochains give rise to steady circular fluid flowing around the chains. Figure 4.3 shows the simulated evolution of the fluids at time $t = 0.2375\text{s}$, indicating rapid liquid mixing by the magnetic nanomixer.

Figure 4.3 2D numerical simulation of localized liquid mixing enhancement by two magnetic nanochains.

4.3.3 Development of DNA and Protein Microarray Assay

DNA and protein microarray were developed to evaluate the assay improvement by the dynamic magnetic nanomixer. A specific nucleic acid sequence¹⁶³ (EC DNA, 50 bp) from *E. coli* O157:H7 and a prostate specific antigen (PSA, 33kD) were chose as targets of the DNA and protein microarray assay, respectively. On the *E. coli* O157:H7 microarray platform, a complementary ssDNA (20 bp, 5'-NH₂ modified) was spotted on an epoxy coated glass slide as the capture probe,¹⁰⁰ and the other complementary ssDNA (20 bp) was terminally labeled with a Cyanine-5 (Cy5) as reporter. In a typical assay, the target DNA was hybridized with the reporter DNA in solutions and finally captured by the complementary DNA immobilized on glass slide (Figure 4.4a). To examine the performance of the *E. coli* O157:H7 microarray, we tested samples of different concentrations of the target DNA and non-complementary DNA (SA DNA from *salmonella* and LM DNA from *Listeria monocytogenes*). The results show the *E. coli* O157:H7 microarray assay has a good linear signal-concentration dependence and an excellent specificity (Figure 4.4b). Similarly, the protein microarray was prepared based on antigen-antibody reaction. In the protein microarray assay, the PSA targets in specimen solutions were recognized by the capture antibodies on the glass slide and the FITC labeled detection antibodies to form sandwich immune complexes (Figure 4.4c).As shown in Figure 4.4d, the spots signal are uniform and linearly correlated with the concentration of PSA.

Figure 4.4 Development of *E. coli* O157:H7 and PSA microarray assay. (a) Schematic illustration of *E. coli* O157:H7 microarray assay based on complementary DNA pairing. (b) *E. coli* O157:H7 DNA microarray assay. (c) Schematic illustration of PSA microarray assay based on sandwich antigen-antibody reaction. (d) PSA microarray assay

4.3.4 Custom-Built Magnetic Stirrer

The rotating magnetic nanochains served as tiny stir bars in sample solutions to promote dynamic mixing, which may greatly accelerate the assay kinetics in a diffusion-limited microarray assay.^{79, 144} Here, we developed a customized magnetic stirrer that can generate a horizontal rotating magnetic field around the microarray.¹⁶⁵ As shown in Figure 4.5, the microarray reaction chamber is placed between two NdFeB permanent magnets that are mounted at the ends of an aluminium turntable and driven by a brushless DC motor. The rotational speed can be continuously adjusted from 0 rpm to over 1000 rpm.

Figure 4.5 (a) Schematic of the custom magnetic stirrer for microarray assay. (b) Picture of the magnetic stirrer.

4.3.5 Optimization of Magnetic Nanomixer Assisted Microarray Assay

To achieve the best mixing effect in microarrays assay, we first examined optimal parameters (such as concentration and rotational speed) of magnetic nanomixers. The DNA/antibodies-spotted slides were assembled with ProPlate frame (16 wells, Grace Bio-Labs) to create separate reaction chambers.^{166, 167} Each chamber has identical probe arrays. A series of samples containing varying concentrations of the magnetic nanomixers (0 to 0.4 mg ml⁻¹) were loaded in the microarray chambers and incubated in the custom magnetic stirrer at rotational speed of 500 rpm. The hybridizations were performed at 42 °C for 1 h. As shown in the Figure 4.6, the fluorescence intensity of the microarray spots increased with increasing concentration of nanomixers and level off at 0.2 mg ml⁻¹.

Figure 4.6 Optimization of the concentration of magnetic nanomixers used for microarray assay.

Then, we further optimized the rotating speed of magnetic nanomixers. Our results (Figure 4.7) demonstrates that the fluorescence intensity increase with the increasing rotational speed of applied magnetic field and drop slightly after 500 rpm. The possible reason for the decline of fluorescence intensity is that the magnetic nanochains cannot maintain synchronous rotation in a rotating magnetic field exceeding 600 rpm,¹⁴⁹ which may make the magnetic nanochains only vibrate rotationally and lead to inefficient mixing. Therefore, magnetic nanomixers at a concentration of 0.2 mg ml⁻¹ and a rotational speed of 500 rpm were eventually chosen for optimized mixing in the microarray assay.

Figure 4.7 Optimization of the rotational speed of magnetic stirrer for microarray assay.

4.3.6 Assay Kinetics Improvement by Dynamic Nanomixers

To evaluate the performance of the magnetic dynamic nanomixers, we compared the assay kinetics of microarray assay in presence and absence of magnetic nanomixers. The assay kinetics were characterized by the fluorescence intensity of the microarray spots with respect to incubation time. For DNA microarray assay, samples containing 2 nM of target DNA (*E. coli* O157:H7 ssDNA) were tested on conventional static and dynamic nanomixer-assisted microarray at incubation time ranging from 10 min to 240 min. The results demonstrate the fluorescence intensity of microspots increases overtime for both assay, and the dynamic mixing led to significantly faster assay kinetics than the conventional static method (Figure 4.8). As shown in Figure 4.8b, the signal generated after 60 min of dynamic mixing is higher than that from 240 min of static assay. It means the dynamic nanomixers can accelerate the assay speed by a factor of 4 or more at the optimal condition. For a desired sensitivity, the

turnaround time of DNA microarray assay can be shorten from multi-hours to less than 1 hour, which is crucial for time-sensitive applications.

Figure 4.8 DNA assay kinetics improvement by dynamic mixing. Representative spots (a) and assay kinetics (b) of DNA microarray assay in presence and absence of dynamic nanomixers. The fluorescence intensity was measured as the intensities of spots signal subtracted by the intensities of local background signal. The error bars represent the s.d. of triplicate measurements ($n=3$).

We further studied the assay kinetics improvement by the magnetic nanomixers in protein (PSA, 20 ng ml⁻¹) microarray assay. The results show the dynamic mixing consistently produces higher signal intensity than static method (Figure 4.9). The PSA microarray assay was speeded up by the magnetic nanomixers from 120 min to less than 60 min.

Figure 4.9 Protein assay kinetics improvement by dynamic mixing. Representative spots (a) and assay kinetics (b) of PSA microarray assay in presence and absence of dynamic nanomixers. Error bars indicate the s.d. of three measurements ($n = 3$).

As given by Stokes-Einstein equation, the diffusion constant is inversely proportional to the particle size.¹⁶⁸ Hence, larger molecule will have higher diffusion limitation, which leads to more pronounced diffusion limitation in assays. Here, we use a 100-bp target DNA to study the effect of dynamic nanomixers. As shown in Figure 4.10, the mixing improvement for assay kinetics is more significant than that of 50-bp target DNA. The signal intensity from static hybridization for 240 min is only comparable to that from dynamic mixing for 30 min, yielding an improvement factor of 8. The results indicate that the dynamic nanomixers perform better in larger molecules assay. In practical

microarray assay, the target nucleic acid sequences are usually more than 1000 bp, which demand an effective mixing method.^{169, 170}

Figure 4.10 Assay kinetics of 100 bp DNA microarray assay in presence and absence of dynamic nanomixers. The error bars stand for s.d. of triplicate measurements ($n = 3$).

4.3.7 Sensitivity Enhancement by Dynamic Nanomixers

In static microarray assay, the vast majority of target molecules in bulk solution cannot effectively bind with the probes on glass surface due to the diffusion-limitation.^{171, 172} As a result, the microarray assay usually suffer from low sensitivity, which limits the application of microarray techniques. To evaluate the contributions of the dynamic mixer of magnetic nanochains to the sensitivity enhancement, we performed a series of dynamic and static (control) microarray assay for comparison. For DNA microarray assay, samples containing different concentration of target DNA (0.5 to 20 nM) were tested in presence and absence of dynamic nanomixers. The results demonstrate that the dynamic

mixing consistently produce stronger signal and higher sensitivity than static control (Figure 4.11). The signal from the static control become nearly indistinguishable when the target concentration decrease to 2 nM. In contrast, the signal from dynamic mixing is detectable (signal intensity: 21.6 ± 3.5) even at a target concentration of 0.5 nM.

Figure 4.11 Representative spots (a) and histogram (b) showing the signal enhancement of DNA microarray assay by dynamic mixing. The error bars represent the s.d. of triplicate measurements ($n=3$).

The similar sensitivity enhancement by dynamic mixing were observed in protein (PSA) microarray assay. As shown in Figure 4.12, the assay sensitivity of dynamic nanomixers assisted PSA assay is 2 ng ml^{-1} (signal intensity: 21.3 ± 4.3), while the assay sensitivity for static control is 10 ng ml^{-1} (signal intensity: 28.3 ± 11.1). It means that the dynamic mixing improved the PSA assay sensitivity from

10 ng ml⁻¹ to 2 ng ml⁻¹.

Figure 4.12 Representative spots (a) and histogram (b) showing the signal enhancement of PSA microarray assay by dynamic mixing. The error bars stand for the s.d. of triplicate measurements ($n = 3$).

4.3.7 Variation Reduction by Dynamic Nanomixers

The increases in the fluorescence intensity and variation reduction by dynamic mixing over that of the static control were summarized in Table 4.1. The signal increase folds are in the range of 1.4 to 5.0, which is comparable to or slightly better than what were produced by other methods.^{15, 23, 115} And we found the signal enhancement by dynamic mixing is more significant at low target concentrations. In addition, the magnetic dynamic nanomixer can promote more homogeneous mixing as they are well dispersed in the bulk solution. The coefficients of variation (CV) among spots were compared between dynamic

mixing and static method (Table 4.1). The results show that the dynamic nanomixers can greatly reduce spot-to-spot variations, thus improving the accuracy of microarray assays. For example, in the low target concentration assay (1 nM of DNA or 5 ng ml⁻¹ of PSA), the CV means is more than 40% for conventional static method. By using dynamic nanomixers, the CV means can be decreased to less than 20%.

Table 4.1 Signal increase (x-folds) and spot-to-spot CV ($n = 6$) variation of the dynamic vs static microarray assays.

EC DNA (nM)	Increase (x- folds)	CV means (%)		PSA (ng ml ⁻¹)	Increase (x- folds)	CV means (%)	
		Static	Dynamic			Static	Dynamic
0.5	--	--	16.3	2	--	--	21.4
1	5.0	43.1	11.1	5	2.6	48.6	17.8
2	3.3	27.9	9.4	10	2.2	35.4	9.6
5	2.4	26.3	4.2	20	2.1	20.3	5.5
10	1.8	19.3	6.0	50	1.9	16.6	6.7
20	1.6	10.1	6.2	100	1.4	14.6	9.5

4.4 Conclusion

In this study, we report the magnetic nanomixers (magnetic nanochains) for improved microarray assays. In the microarray analysis, the magnetic nanochains are premixed with liquid samples and actuated by a rotating magnetic field. The rotating magnetic nanochains in sample solutions serve as tiny stir bars to promote dynamic mixing and effectively accelerate the transportation of targets to the vicinity of the probes immobilized on glass surface. The rapid mixing minimizes the localized target depletion, resulting in rapid turnaround, improved sensitivity and reduced spot-to-spot variation. We fabricated a custom magnetic stirrer and developed the DNA/protein microarray assays to evaluate the performance of magnetic nanomixers. We demonstrated that the dynamic nanomixers considerably increased the assay kinetics and signal intensity in both DNA and protein microarray assay. The CV of spots was reduced by dynamic mixing. The magnetic nanomixer assisted microarray assays are simple, cost-effective, and compatible with any commercial microarrays. The magnetic dynamic nanomixers were expected to be used in other on-surface bioassays that suffer from diffusion limitation, such as microfluidic assays, enzyme-linked immuno sorbent assay (ELISA), and heterogeneous catalysis.

Chapter 5 Magnetic Nanochain Integrated Microfluidic Biochips (MiChip)

5.1 Introduction

Microfluidic systems that offer precise control of fluids, low sample and reagent consumption, and rapid sample processing are of considerable interest for the development of miniaturized, portable and low-cost analytical platforms^{26, 27}. In particular, the identification and quantification of molecular and cellular targets using microfluidic biochips are under intense research for a wide spectrum of applications ranging from fundamental biology to clinical diagnostics^{117, 119, 122}. Current designs of microfluidic biochips commonly contain built-in components such as sensing element-functionalized surfaces and liquid mixers^{32, 124, 173}. Biofunctionalized surfaces serve to separate and enrich targets of interest from complex fluid samples, which is key to specific detection in subsequent signal transduction^{129, 130}. On the other hand, spatial confinement in microchannels leads to low Reynolds number fluids under laminar flow, which causes inefficient mixing across the channels mainly controlled by diffusion.^{35, 174} Hence, passive or active mixers are introduced to enhance on-chip liquid mixing and mass transfer, which is critical for improving kinetics and sensitivity of the diffusion-limited on-surface assays in microchips¹²⁶⁻¹²⁸. However, despite recent success in the laboratory-scale demonstration of microfluidic bioanalysis, these necessary built-in components inevitably increase structural, fabricating,

operational and translational complexity of the chips. It remains challenging to realize integrated liquid mixing, bioseparation and signal transduction in simple microfluidic configurations.

Here we report a new type of broadly applicable multiplexing microfluidic biochips based on bioconjugated magnetic nanochains (Magchains). Our studies demonstrated that the Magchains can be actuated by tailored magnetic fields to serve as nanoscale stir bars to promote rapid active on-chip liquid mixing and capture agents for targets of interest, leading to integrated liquid mixing, bioseparation and signal transduction. As a result, the Magchain-integrated microchips (MiChip) can adopt a simple planar design consisting of flat channels free of any built-in target-specific components, such as sensing elements-functionalized surfaces and liquid mixers that are commonly found in conventional biochips. The MiChip is therefore broadly applicable for a diverse range of targets. Furthermore, in contrast to the inefficient target capture at liquid-solid interfaces in conventional designs, the use of well-dispersed nanochains under continuous mixing overcomes the problem associated with diffusion-limited assay kinetics, giving rise to a rapid turnaround time.

Raman spectroscopy was employed in this study for on-chip detection because of its tolerance towards complex, unprocessed liquid samples, excellent compatibility with microfluidic devices, high spatial resolution, and superior photostability of Raman probes.^{55, 175-177} Plasmonic nanostructures with surface-confined electromagnetic field are able to greatly amplify, by an enhancement

factor (EF) of 10^5 or more, the intrinsically weak Raman scattering of molecules in close proximity.^{178, 179} Anchoring Raman reporter molecules of different chemical structures on plasmonic metal nanostructures leads to SERS probes with distinct, sharp spectroscopic signatures that can be excited and differentially detected simultaneously, making them excellent multiplexing tags.^{54, 180, 181}

5.2 Experimental Section

5.2.1 Materials

Chemicals. Dopamine hydrochloride, Iron(II) chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), Iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), Gold (III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), silver nitrate (AgNO_3), sodium chloride (NaCl), trisodium citrate, sodium acetate, ethylene glycol, sodium boronhydride (NaBH_4), cetyltrimethylammonium bromide (CTAB), Tris(hydroxymethyl)aminomethane (TRIS), hydroquinone, bicine, sodium dodecyl sulfate (SDS), MES hydrate, ethyl dimethylaminopropyl carbodiimide (EDC), sulfo-Nhydroxysuccinimide (NHS), bovine serum albumin (BSA), human serum albumin (HAS), 4-Nitrothiophenol (4-NTP), 4-bromothiophenol (BTP), 2,3,5,6-tetrafluorothiophenol (TFTP), 3,5-difluorothiophenol (DFTP), 2,4-dichlorothiophenol (DCTP), and 4-methoxy- α -toluenethiol (MATT). 3,5-bis(trifluoromethyl)benzenethiol (FMBT), 2-fluorothiophenol (2-FTP), 3-fluorothiophenol (3-FTP) were purchased from Sigma Aldrich. *N,N*-Dimethylmethanamide (DMF) were purchased from Fisher Chemical. Thiolated methoxy-poly(ethylene) glycol (mPEG-SH, 5 kDa) and thiolated caboxy-poly(ethylene) glycol (HS-PEG-COOH, 5kDa) was purchased from Laysan Bio, Inc. Su-8 100 was purchased from MicroChem. Sylgard[®] 184 silicone elastomer kit was obtained from Dow Corning.

Biomaterials. All the standard proteins (PSA, CEA, and AFP) were purchased from Fitzgerald: PSA (30C-CP1017), CEA (30-1819), and AFP (30-

1029)). The following antibodies were used in this study: capture & detection antibody to PSA (GenScript), capture & detection antibody to CEA, and AFP (Meridian, Life Science, Inc.), capture & detection antibody to *E. coli* O157:H7 (generated and provided by Dr. Weihua Lai), capture & detection antibody to *S. aureus* (Thermo Fisher). All bacteria were purchased from the American Type Culture Collection (ATCC). The bacterial strains used in the study were *E. coli* O157:H7 (ATCC 43888), *S. aureus* (ATCC 25923), *E. coli* O6 (ATCC 25922), *S. enterica* (ATCC 13311), *P. aeruginosa* (ATCC 142) and *E. faecalis* (ATCC 29212).

5.2.2 Bacteria Culture

Bacteria were seeded and cultured in suspension using the following media: *E. coli* O157:H7, *E. coli* O6, *S. enterica*, and *P. aeruginosa* were cultured in Luria–Bertani medium (LB, Oxoid, Basingstoke, UK) at 37 °C for 20 h; *S. aureus* and *E. faecalis* were cultured in Brain-Heart infusion broth (Oxoid, Basingstoke, England) at 37 °C. The concentration of *S. aureus* was determined by serial dilution with subsequent plating on agar plates and measurement of colony forming units (CFU). The cells were then treated with 0.3% formaldehyde for 24 h. The inactivated bacteria were collected by centrifugation at 4000 rpm and resuspended in 0.01 M PBS (pH 7.4). Finally, these bacteria were serially diluted to the desired concentrations with 0.01 M PBS (pH 7.4) for further use.

5.2.3 Serum Samples

The study was approved by the ethics committee of Tongji Medical College, Huazhong University of Science and Technology (IORG No. IORG0003571), and informed consent was obtained from all participants. Blood samples were collected from patients with prostate cancer ($n = 10$), hepatocellular carcinoma ($n = 3$) and colorectal cancer ($n = 7$). Serum was obtained via venipuncture into vacutainer tubes containing clot activator (BD Diagnostics, Franklin Lake, NJ). Cancer diagnoses were confirmed by traditional examination at Cancer Center, Union Hospital, Tongji Medical College.

5.2.4 Preparation of Antibody-Conjugated Magnetic Nanochains

The magnetic nanochains were synthesized according to the method in Section 3.2.3. The harvested nanochains were washed 3 times with DI H₂O and dispersed in 5 ml of bicine buffer (pH 8.5, 10 mM). 100 μ l of antibody solution (Ab-I, 0.25 mg ml⁻¹) was added to the dispersion and the mixture was shaken for 12 h at room temperature. Afterwards, 0.5 ml of mPEG-SH solution (10 mg ml⁻¹) was added to the mixture to block the unreacted sites for 12 h. The excess reactants were removed by repeated magnetic separation and washing. Finally, the Magchains were resuspended in 1 ml PBS (0.01 M, pH 7.4) and stored at 4 °C before use.

5.2.5 Synthesis of Gold Nanorods (AuNRs)

A seed-mediated method was used to prepare the AuNRs.¹⁸² First, 0.60 ml of ice-cold NaBH₄ (10 mM) was quickly added to 10 ml of HAuCl₄ (0.25 mM) dissolved in 0.2 M CTAB surfactant solution. The mixture was stirred for 2 min, producing a brownish yellow solution of small gold seeds. The seed solution was kept for at least 1 h at 30 °C before it was used in the next step. Second, AuNRs were synthesized in a growth solution. 40 µl of AgNO₃ (0.1 M) was added to 10 ml of HAuCl₄ solution (1.25 mM) in 0.1 M CTAB. 0.5 ml of hydroquinone aqueous solution (0.1 M) was then introduced to reduce HAuCl₄ to colorless HAuCl₂, followed by the injection of 0.16 ml of seed solution to initiate the growth of AuNRs. The growth solution was mixed thoroughly for 2 min and kept undisturbed in a water bath at 30 °C for 12 h. The AuNRs were purified three times by centrifugation (5000 rpm, 30 min) and were dispersed in 10 ml of DI H₂O.

5.2.6 Preparation of SERS-encoded AuNRs

The AuNRs were encoded with different Raman reporter molecules using the following procedure. First, 2 ml of AuNRs solution was centrifuged at 5000 rpm for 10 min and redispersed in 2 ml of dimethyl formamide (DMF). Then, 200 µl of Raman reporter molecules (20 mM in DMF) were added to the solution and the reaction mixture was stirred for 2 h. In this process, Raman molecules

anchored on the surface of AuNRs via the Au-S bond. The SERS-encoded AuNRs were then harvested by centrifugation at 5000 rpm for 10 min and redispersed in 2 ml of fresh DMF. A mixture of mPEG-SH (50 μ l, 1 mM, MW= 5000) and HOOC-PEG-SH (300 μ l, 1 mM, MW= 3400) was added to the mixture and reacted for 1 h, resulting in a mixed layer of mPEG-SH and HOOC-PEG-SH on the AuNRs surface. Finally, the PEGylated AuNRs were centrifuged twice at 5000 rpm for 10 min and resuspended in 2 ml of DI H₂O.

5.2.7 Preparation of Antibody-Conjugated SERS Probes

The harvested SERS-encoded AuNRs were conjugated with different antibodies using a similar protocol. Briefly, the carboxyl functionalized AuNRs were collected by centrifugation and dispersed in 4 ml of MES buffer (0.01 M, pH 5.5). Next, 0.2 ml of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 5 mg ml⁻¹) and sulfo-N-hydroxysuccinimide (sulfo-NHS, 5 mg ml⁻¹) were quickly added and incubated for 30 min to activate the carboxylic acid groups. The excess EDC and sulfo-NHS was removed by centrifugation, and 20 μ g of detection antibody (Ab-II) in 2 ml of borate saline buffer (0.01 M, pH 8.5) was introduced immediately to the activated particles and the mixture was reacted under gentle stirring for 3 h at room temperature. Finally, 0.2 ml of bovine serum albumin (BSA) solution (10 mg ml⁻¹) was introduced to the mixture for surface blocking. After 1 h, the SERS probes were centrifuged to separate the free reactants.

5.2.8 Fabrication of Microfluidic Chips

The microfluidic chips (single channel unit and multichannel arrays) were fabricated according to the method in Section 3.2.5. After fabrication, the internal surfaces of the microfluidic chips were PEGylated to suppress potential biofouling by non-specific constituents in liquid specimens. At first, the dopamine solution (1 mg ml^{-1} in pH8.5 bicine buffer) was injected into the microfluidic channels at room temperature for 4 h. Then the channels were washed with DI H₂O for 2 times. After that, the mPEG-SH solution (5 mg ml^{-1} in pH8.5 bicine buffer) was injected into the microfluidic channels at room temperature for 12 h. Finally, the internal surface PEGylated microfluidic chips were washed with DI water before use.

5.2.9 Electromagnetic Coils Setup

The working principle of the electromagnets providing a rotating magnetic field is similar to a two phase brushless DC motor. The four coils are mounted on a cross-shaped aluminum plate. Each coil consists of windings maximizing the space allowed by a quadrant on the plate. Ferrite cores were inserted in the coils to increase the magnetic flux. A function generator supplies the reference pulse trains for a stepper motor driver to generate the two phases of high current excitation at a microstepping setting of 1/128 to approximate a sinusoidal waveform. The coils generate a rotating magnetic field with adjustable

frequencies in the range of 1 - 11 Hz (60 - 660 revolutions per minute).

5.2.10 Detection of Biomarkers on the MiChip

For the detection of cancer protein biomarkers, 1 μl of standard samples (0.1 to 100 ng ml^{-1}) or clinical serum samples and 1 μl of detection reagents (Magchains and SERS probes) were injected into the MiChip platform. The two streams were then mixed within the mixing chamber, and followed by target recognition, separation and Raman measurements. Similarly, bacteria of a range of concentrations (10^0 to 10^4 $\text{CFU } \mu\text{l}^{-1}$) and bacteria spiked saliva were tested using the MiChip platform. All measurements were done in triplicate, and data are shown as mean $\pm$ SD.

5.2.11 Enzyme-linked immunosorbant assay (ELISA)

The concentration of cancer biomarkers (PSA, CEA and AFP) was measured using commercial ELISA kits: PSA (IB19126, IBL-America), AFP (IB19102, IBL-America), and CEA (RAB0411, Sigma-Aldrich). The assays were conducted according to instructions for use. Briefly, 25 μl of standards, controls and unknown samples were introduced to selected wells. Afterwards, 100 μl of conjugate reagent was added to all wells and incubated for 30 min at room temperature. The wells were subsequently washed three times with 0.01% Tween-20 in PBS (PBST, $\text{pH}=7.4$), and horseradish peroxidase (HRP)-conjugated secondary antibody was added to all wells and incubated for 30 min.

The reaction wells were then washed five times with PBST followed by 15 min incubation in 100 μ l of 3,3',5,5'-tetramethylbenzidine reagent. Finally, all wells were supplemented with 50 μ l of stop solution (2 M H₂SO₄) and analysed for absorbance at 450 nm.

5.2.12 Preparation of Raman Reporters Embedded Au@Ag SERS Probes

A wet-chemical approach was used to prepare high quality Au@Ag SERS probes based on plasmonic metal nanoparticles.^{183, 184} Typically, 50 μ l of CTAC capped AuNPs was added into 3 ml of DMF under stirring. Subsequently, 50 μ l of 4-NTP (or other Raman reporters) was added in the solution under stirring for 3 h. Then, 300 μ l of Polyvinyl Pyrrolidone solution (PVP, 10% in DMF) and 300 μ l of hydroquinone were added in the mixture under stirring. Afterwards, 1.25 ml of AgNO₃ solution (2 mM in H₂O) was dropwise added in the mixture within 1 h. The mixture was kept stirring for 2 h to allow the Ag deposition on the surface of AuNPs. A mixture of mPEG-SH (50 μ l, 1 mM, MW= 5000) and HOOC-PEG-SH (300 μ l, 1 mM, MW= 3400) was subsequently added to the mixture and reacted for 1 h. The PEGylated Au@Ag core shell nanoparticles were centrifuged twice at 5000 rpm for 10 min and resuspended in 2 ml of DI H₂O. Finally, the harvested 4-NTP embedded Au@Ag NPs were conjugated with different antibodies according to the method in Section 5.2.7.

5.2.13 Characterization analysis

Scanning electron microscopy (SEM) images were captured using a field-emission scanning electron microscope (JSM-6700F). Transmission electron microscopy (TEM) observations were conducted on a Jeol JEM 2010 electron microscope at an acceleration voltage of 300 kV. SERS experiments were conducted using a Renishaw Raman microscope equipped with 633/785 nm excitation lasers. The laser beam with a laser spot size of 2 - 5 μm was focused by a 50 $\times$ objective. A single scan with an integration time of 15 s was performed for each spectrum acquisition.

5.3 Results and Discussion

5.3.1 Design of the MiChip

Figure 5.1 illustrates the design of the MiChip. As shown in Figure 5.1a and Figure 5.1b, the basic unit of the polydimethylsiloxane (PDMS)-on-glass MiChip platform features a mixing chamber, a detection chamber, four fluid ports for sample input and waste output, and two pneumatic microvalves that control the fluid delivery into/from the mixing chamber. Importantly, the simple design of this basic unit can be easily expanded into integrated multichannel arrays for parallel analysis of multiple specimens. Figure 5.1c shows a model of a 4-channel system, in which the arrayed units share a common waste outlet and pneumatic microvalves. The individual unit can be operated independently without crosstalk, which is critical for high-throughput screening.

Figure 5.1 Design of the magnetic nanochain integrated microfluidic chip

(MiChip). (a) Schematic illustration of the MiChip assay platform. Photographs of the single channel unit (b) and multichannel arrays microfluidic chip (c). The microchannel was filled with a red dye for better visualization. Scale bar, 0.5 cm. Inset of (b): microscopy image of detection chamber, 50 x magnification.

The dimensions of each part of the chip are shown in Figure 5.2a. The chambers and channels have a uniform height of 50 μm , with internal surfaces of the MiChip PEGylated to suppress potential biofouling by non-specific constituents in liquid specimens. Of particular note is that the MiChip adopts a simple planar design consisting of flat channels and is free of any target-specific components (Figure 5.2b).

Figure 5.2 (a) Design of the single assay microfluidic chip. The dimensions of channels and chambers are labeled. (b) The bright field images of mixing chamber and detection chamber.

5.3.2 On-Chip Detection of Targets

In the MiChip assay, antibody-conjugated magnetic nanochains (Magchain) are directed by the external magnetic field to concurrently drive sample mixing and target capture/enrichment. SERS-encoded nanoprobe (SERS probe) serve

as ultrasensitive signaling probes to realize multiplexed detection. The MiChip assay is streamlined into three key steps (Figure 5.3): First, unprocessed liquid specimen (1.0 μl) and a mixture of Magchains and SERS nanoprobe in PBS buffer (1.0 μl) are injected into the chip via the two inlets, respectively. Second, the liquid delivered into the mixing chamber is confined inside by the pneumatic microvalves 1 and 2, and undergoes mixing driven by the synchronized rotation of the Magchains in a spinning magnetic field generated by four orthogonal electromagnetic coils. During this process, antigen-antibody recognition results in sandwich immune complexes of the Magchains, the targets of interest and the SERS probes in the chamber. Third, microvalve 2 is opened and all the Magchains including those in the immune complex are magnetically collected from the mixing chamber into the detection chamber for Raman spectroscopic detection. The volume difference of mixing and detection chambers leads to an enrichment factor of 100.

Figure 5.3 Schematic illustration of the MiChip assay for the detection of

biomarkers.

A picture of the whole setup including the microfluidic chip and the electromagnetic coils is presented in Figure 5.4. Since we need to monitor the rotation and enrichment of the Magchain in this study, it was mounted on a microscope equipped with a CCD and a spectrometer.

Figure 5.4 Schematic of the MiChip assay setup including the picture of the microfluidic chip and the electromagnetic coils.

5.3.3 Synthesis of Gold Nanorods

Gold nanorods (AuNRs) were synthesized by using hydroquinone as reducing agent.¹⁸² The results show the aspect ratio and the longitudinal surface plasmon resonance (LSPR) wavelength of AuNRs can be controlled by changing the gold chloride concentration (Figure 5.5). In this study, AuNRs with LSPR centered at 795 nm were used as the substrates for SERS-encoded nanoprobe to achieve efficient excitation and sensitive detection at near-infrared (NIR)

wavelengths (*i.e.*, 785 nm), where the absorption and scattering by complex fluids such as blood is minimal.¹⁸⁵

Figure 5.5 SEM images (a-d) and LSPR (e) of AuNRs synthesized at different concentration of HAuCl₄: 0.75 mM, 1.0 mM, 1.25 mM, 1.5 mM.

5.3.4 Preparation of SERS Nanoprobes

As illustrated in Figure 5.6, Raman reporters were introduced to anchor on the AuNRs surface via Au-S bond. Subsequently, mPEG-SH and HS-PEG-COOH were grafted on the Raman molecules-encoded AuNRs. Finally, the detection antibody (Antibody-II) was conjugated with the carboxylic acid groups at the end of the PEG chains via the EDC/NHSS coupling reaction.

Figure 5.6 Schematic illustration of the preparation of SERS-encoded nanoprobe in stepwise anchoring of Raman reporter molecules on the AuNRs, PEGylation, and antibody conjugation.

We first compare the SERS spectra of all 4 AuNRs with different LSPR wavelength from Section 5.3.3. The results (Figure 5.7) show that the AuNRs with a longitudinal LSPR at 795 nm has the highest SERS signal, which is consistent with our choice of SERS nanoprobe.

Figure 5.7 (a) SERS spectra of 4 representative NTP-encoded AuNRs with different longitudinal LSPR. (b) SERS intensity at 1341 cm^{-1} of the NTP-encoded AuNRs with different LSPR peaks.

Different Raman molecules (4-nitrothiophenol, 4-bromothiophenol, 2,3,5,6-tetrafluorothiophenol, 3,5-difluorothiophenol, 2,4-dichlorothiophenol, and 4-

methoxy- α -toluenethiol) of distinct vibrational profiles were encoded on the same AuNRs via Au-sulfur bond. The resultant SERS encoded nanoparticles have the same size but molecule-defined spectral signatures that can be excited at a single wavelength, detected simultaneously, and easily distinguished from each other (Figure 5.8), offering unique advantages for multiplexed detection.⁵¹

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Figure 5.8 (a) SERS spectra of 6 representative SERS-encoded AuNRs. From bottom to top: 4-nitrothiophenol (NTP), 4-bromothiophenol (BTP), 2,3,5,6-tetrafluorothiophenol (TFTP), 3,5-difluorothiophenol (DFTP), 2,4-dichlorothiophenol (DCTP), and 4-methoxy- α -toluenethiol (MATT).

Then we investigated the dispersibility of AuNRs after Raman molecules anchoring, PEGylation, and antibodies conjugation, since the aggregation will seriously affects the quality of SERS probe. LSPR peaks of plasmonic nanostructures are highly sensitive to interparticle distances, experiencing significant red-shifts upon aggregation. UV-vis (Figure 5.9a) and Raman spectra (Figure 5.9b) reveal that the three steps of surface functionalization of the AuNRs

(length/width= 100/28 nm; LSPR~ 795 nm) did not cause significant changes in their LSPR peaks and SERS profiles, indicative of the presence of stable individual AuNRs (TEM image in Figure 5.9a), which is crucial for their utilization in quantitative analysis.

Figure 5.9 (a) UV-vis spectra of original AuNRs, SERS-encoded AuNRs and antibody-conjugated SERS probes. Inset: TEM image of the antibody-conjugated SERS probes. Scale bar, 100 nm. (b) Characterization of SERS probe. From bottom to top: Raman spectra of naked AuNRs, SERS-encoded AuNRs and antibody-conjugated SERS probes.

The stability of SERS probes in high salt or protein solution was also investigated.

The results show the naked AuNRs were aggregated in the electrophoretic buffer solution and the PEGylated SERS probes are stable even in high salt and protein solutions (Figure 5.10), making the PEGylated SERS probes suitable to use in complex clinical samples.

Figure 5.10 Agarose gel electrophoresis analysis of the stability of SERS probes. Lane 1, original AuNRs. Lane 2, PEGylated SERS probes. Lane 3-5, PEGylated SERS probes that incubated with 0.01 M of PBS (lane 3), 5% of NaCl (lane 4), and 10% of BSA (lane 5) for 0.5 h.

5.3.5 Multiplexed Detection of Protein Biomarkers

In the first proof-of-concept application, we explored the use of the MiChip platform for multiplexed detection of three serum protein biomarkers: prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), and α -fetoprotein (AFP), which are commonly used clinical biomarkers for prostate, colorectal and hepatocellular cancers, respectively^{187, 188}. Technologies that allow for sensitive, timely measurement of multiple biomarkers in small volume of specimens hold great promise for screening, early detection, and outcome analysis of complex diseases such as cancer. Respective capture antibody on the Magchains and detection antibody on the SERS probes for the targets were identified to minimize cross-reactivity. The Magchains for multiplexed assays are modified to carry a nearly equal number of all three capture antibodies (Figure 5.11). In our previous

study (Section 3.3.8), we have demonstrated the multiplexed nanochains can simultaneously capture the three targets with a high capture efficiency (85-88%) and with low crosstalk against the mismatched targets (< 5%). The high recovery rate and negligible crosstalk of Magchain-based bioseparation are key prerequisites to realize multiplexed assays.

Figure 5.11 Conceptual illustration of the simultaneous detection of PSA (green), AFP (blue), and CEA (red).

We selected Raman reporters, *i.e.*, 4-nitrothiophenol (NTP), 4-bromothiophenol (BTP), and 2,3,5,6-tetrafluorothiophenol (TFTP) to encode PSA, AFP, and CEA, respectively. Our previous result in Section 3.3.8 confirms that 5 min of mixing captures more than 80% of PSA (100 ng ml⁻¹) and all of the Magchains can be collected into the detection chamber within 1.5 min. Therefore, the streamlined assay is set to complete within 8 min, fractioned into 5 min of Magchain-driven mixing, 1.5 min for magnetic separation, and 1.5 min for liquid transport and spectroscopic readout. The results of the MiChip assay with PSA

standards show that *in-situ* reading of SERS intensity at 1341 cm^{-1} of NTP is linearly proportional to PSA concentration in the range of $0.1\text{--}100\text{ ng ml}^{-1}$ (Figure 5.12). The relative standard deviation of the SERS signal remains below 6% in the entire concentration range, indicating good statistical accuracy.

Figure 5.12 (a) SERS spectra of different concentrations of PSA in PBS (ranging from 0 to 100 ng ml^{-1}). (b) The SERS intensity at 1346.5 cm^{-1} as a function of the concentration of PSA. Insets: calibrations curves at low concentrations (ranged from 0 to 0.1 ng ml^{-1}).

The MiChip design allows for easy recovery of separated targets via the detection chamber. As revealed by scanning electron microscopy (SEM) observation, more AuNRs are found on the nanochains (Figure 5.13) recovered from the standard samples with higher PSA concentrations, in line with the SERS results. The control sample without PSA, on the other hand, showed no AuNR on the nanochain surfaces, confirming the high specificity of our design.

Figure 5.13 SEM images of Magchain-PSA-SERS probes complex formed at varying concentrations of PSA. Scale bar, 500 nm.

What's also important is that SERS measurements in various control media showed minimal non-specific binding (Figure 5.14). Besides the sample volume ($\sim 1 \mu\text{l}$) defined by the capacity of the mixing chamber, we optimized and fixed all the assay conditions in sample (Magchains and SERS probes) loading, magnetic mixing and separation, and spectral acquisition to achieve comparable quantitative analysis..

Figure 5.14 Specificity of the MiChip assay. The assay shows minimal non-specific binding caused by PBS, 5% BSA, 1% glucose and cell culture medium

(10% FBS).

Indeed, AFP and CEA measurements showed similar trends of linear signal-concentration dependence (Figure 5.15). Detection limit of the on-chip measurement is $\sim 10 \text{ pg ml}^{-1}$ (insets in Figure 5.15b and 5.15d), which is two orders of magnitude lower than that ($\sim 1.0 \text{ ng ml}^{-1}$) of commercial ELISA kits.

Figure 5.15 SERS spectra of AFP (a) and CEA (c) at varying concentrations (ranging from 0 to 100 ng ml^{-1}) and the SERS intensity at 1070.7 cm^{-1} and at 884.9 cm^{-1} as a function of the concentration AFP (c) and CEA (d), respectively.

The well-resolved peaks at 1341 , 1071 , and 885 cm^{-1} of the three SERS probes allow for readily simultaneous detection of their encoded biomarkers. The number of Raman molecules on AuNRs was controlled to afford similar SERS intensity for the same concentration of biomarkers, which enabled

straightforward multiplexed quantification of the biomarkers in a series of samples containing varying concentrations of the target(s) (Figure 5.16).

Figure 5.16 SERS responses to mixtures of the three cancer biomarkers (PSA, AFP and CEA) with various ratios.

5.3.6 Michip Assay of Clinical Samples

We further examined the clinical utility of the MiChip assay by measuring the same panel of biomarkers in serum samples from 20 cancer patients diagnosed with prostate ($n=10$), colorectal ($n=7$) and hepatocellular ($n=3$) cancers. The current biomarker calibration range ($0.1\text{--}100\text{ ng ml}^{-1}$) evaluated by the MiChip assay is clinically relevant. For example, the commonly used serum cutoff values of PSA, AFP, and CEA are 4, 10 and 5 ng ml^{-1} , respectively¹⁸⁹⁻¹⁹¹. Simulated serum samples containing equal concentrations of PSA, AFP, and CEA in the range of $0\text{--}100\text{ ng ml}^{-1}$ were subjected for on-chip multiplexed analysis to establish calibration curves for each target. The SERS intensity at

specific peaks retained its linear dependence on biomarker concentration (Figure 5.17) even under the multiplexed detection conditions.

Figure 5.17 (a) SERS responses to a mixture biomarkers (PSA:AFP:CEA=1:1:1, ranging from 0 to 100 ng ml⁻¹) spiked in artificial serum samples. (b) Standard curves for the multiplexed quantitative analysis were generated by plotting the SERS peak intensity at 1341, 1071 and 885 cm⁻¹ against the concentrations of PSA, AFP and CEA, respectively.

The levels of the three biomarkers in the 20 clinical specimens were measured by the MiChip assay and compared with results measured by standard ELISA commercial kit (Figure 5.18). The excellent linear correlation ($R^2 = 0.996$) of the ELISA and MiChip results (Figure 5.19) confirm the ability of the MiChip platform to analyse complex clinical specimens.

Importantly, the results showed that the MiChip assays were able to distinguish the three groups of cancer patients without any exception (Figure 5.18). For example, PSA concentrations varied from 15.3 to 58.1 ng ml⁻¹ for the

prostate cancer patients (P11-P20) with a mean of 30.2 ng ml^{-1} . The PSA level of the colorectal and hepatocellular cancer patients fell into the range of 0.8 to 1.4 ng ml^{-1} . PSA tests identified all the prostate cancer patients with good statistical accuracy ($P < 0.0001$). The results on detection of AFP ($P < 0.0001$) and CEA ($P = 0.0003$) are also consistent with the finding with PSA.

Figure 5.18 The levels of the three biomarkers in the 20 clinical specimens measured by the MiChip assay and ELISA.

Figure 5.19 Correlation between data obtained from the MiChip assay and ELISA for the concentrations of PSA, AFP, and CEA in 20 serum samples from cancer patients.

5.3.7 Rapid and Multiplexed Detection of Bacteria

We next extended the MiChip assay to multiplexed identification and quantification of bacterial species (Figure 5.20). The rapid and sensitive detection of multiple infectious species such as bacteria is of considerable public interest for pinpointing the source of infection at the early stage and improving patient care and outcomes^{29, 122}. Magchains and SERS probes were prepared as described above. Here, the Raman reporter molecules, 3,5-difluorothiophenol (DFTP) and 2,4-dichlorothiophenol (DCTP) were chosen to prepare SERS probes specific to *Escherichia coli* O157:H7 (*E. coli* O157:H7) and *Staphylococcus aureus* (*S. aureus*) bacteria.

Figure 5.20 Schematic illustration of the simultaneous detection of *E. coli* O157:H7 (blue) and *S. aureus* (purple)

SEM images of samples collected from the detection chamber show that *E.coli* O157:H7 cells (Figure 5.21a) and *S. aureus* (Figure 5.21b) were conjugated with multiple Magchains and even more with SERS probes.

Figure 5.21 SEM images of Magchains-bacteria-SERS probes complex with varying concentrations of *E. coli* O157:H7 (a) and *S. aureus* (b). Scale bar, 1 μm .

For saliva samples containing *E. coli* O157:H7 (Figure 5.22a) or *S. aureus* (Figure 5.22b) or both (Figure 5.22c), the SERS signals at the characteristic peaks (1001.6 cm^{-1} for *E. coli* O157:H7 and 1104.9 cm^{-1} for *S. aureus*) show consistent linear correlation with the bacterial concentration over four orders of magnitude ranging from 10^0 to $10^4\text{ CFU }\mu\text{l}^{-1}$ (insets in Figure 5.22a and 5.22b). Given the capacity ($1\text{ }\mu\text{l}$) of the mixing chamber, this means that the MiChip assay offers a sensitivity that allows for detecting a single bacterium. Furthermore, screening among a panel of Gram-positive and Gram-negative bacteria ($10^2\text{ CFU }\mu\text{l}^{-1}$) led to strong signals from specific types of targets and negligible background from non-specific ones (Figure 5.22d), demonstrating the high specificity for bacteria identification.

Figure 5.22 SERS spectra of *E. coli* O157:H7 (a), *S. aureus* (b) and both (c) at different concentrations (ranging from 0 to 10^4 CFU μl^{-1}). (d) Specificity of MiChip assay of *E. coli* O157:H7, *S. aureus* and other control bacteria spiked saliva specimen. The error bars represent the s.d. of triplicates ($n = 3$).

The identification of infectious sources among a library of pathogenic species is critical for the selection of effective treatments. The MiChip assay can be easily customized into integrated kits for sensitively screening a panel of pathogens leading to similar symptoms within 8 min. In contrast, the slow turnaround of the current gold standard procedure such as bacterial culture remains a major challenge.

5.3.8 Sensitivity Improvement by Using Raman Molecules Embedded

Au@Ag Core Shell Nanoprobes

The MiChip platform offers versatile opportunities to further extend its use for a broader range of targets and improve its sensitivity. Recent advances in nanochemistry have opened the avenue to systematically optimize the spectral and structural details of plasmonic nanostructures for further improving the Raman enhancement efficiency and thus the sensitivity of the MiChip assay.¹⁹²⁻¹⁹⁵ For example, the use of 4-NTP-embedded Au@Ag core-shell nanoprobe¹⁸⁴ (Figure 5.23) that exhibits 40 folds of Raman intensity of the AuNR probe. The use of 4-NTP-embedded Au@Ag core-shell nanoprobe is able to push the limit down by nearly 2 orders of magnitude (Figure 5.24), achieving 0.2 pg ml⁻¹ for PSA.

Figure 5.23 (a) Schematic illustration of the synthesis of Raman tags (*i.e.*, 4-NTP) embedded SERS probes. (b) TEM images of AuNPs@NTP@Ag. (c) SERS spectra of AuNRs@NTP (1 nM) and AuNPs@NTP@Ag (0.1 nM).

Figure 5.24 (a) MiChip assay of PSA by using AuNPs@NTP@Ag nanoprobe. (b) Standard curve for the quantitative analysis were generated by plotting the SERS peak intensity at 1341cm^{-1} against the concentrations of PSA. Inset: calibrations curve for PSA concentration ranged from 0 to 1 pg ml^{-1} .

5.4 Conclusion

Microfluidic biochips hold great potential for liquid analysis in biomedical research and clinical diagnosis.¹⁹⁶⁻¹⁹⁸ However, the lack of integrated on-chip liquid mixing, bioseparation and signal transduction presents a major challenge in achieving rapid, ultrasensitive bioanalysis in simple microfluidic configurations. Here we reported magnetic nanochain integrated microfluidic chip built upon the synergistic functions of the nanochains as nanoscale stir bars for rapid liquid mixing and as capturing agents for specific bioseparation. The use of magnetic nanochains enabled a simple planar design of the microchip consisting of flat channels free of common built-in components, such as liquid mixers and surface-anchored sensing elements. The microfluidic assay, using surface-enhanced Raman scattering nanoprobes for signal transduction, allows for streamlined parallel analysis of multiple specimens with greatly improved

assay kinetics and delivers ultrasensitive identification and quantification of a panel of cancer protein biomarkers and bacterial species in 1 μ l of body fluids within 8 min.

5.5 Declaration

The work presented in this chapter has been published in *Nature Communications*.

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Chapter 6 Conclusions and Future Research

6.1 Conclusions

In this thesis, we successfully developed a novel of dual-functional magnetic nanochains based on the adhesive and versatile reactivity properties of polydopamine. The bioconjugated magnetic nanochains can be actuated by tailored magnetic fields to serve dual-functional roles as both nanoscale stir bars and capture agents, promoting concerted active liquid mixing and bioseparation. We first investigated the performance of magnetic nanochains in mixing and bioseparation. The obtained magnetic nanochains then have been used in microarray and microfluidic bioassay.

First, we studied the rotating characteristic of magnetic nanochains in response to an alternating magnetic fields with varying rotational speed. We demonstrated the magnetic nanochains can keep synchronous rotation at the maximum frequency of 540 rpm. At the speed of 500 rpm, the optimized magnetic nanochains demonstrated a high mixing efficiency (~80%) within 1 min. Under continuous mixing, the antibody conjugated magnetic nanochains have shown high capture rate (>80%) even against multiple targets in 5 min.

Second, the PEGylated magnetic nanochains were applied in DNA and protein microarray assay as dynamic nanomixer to improve the performance of

microarray assay. Our results have shown that the use of magnetic nanomixers led to improved kinetics, increased sensitivities, and reduced signal variation in both of DNA and protein microarray analysis. In comparison with exiting methods, our magnetic nanomixer based approach offers rapid turnaround, improved sensitivity, good accuracy, low cost, simple operation, and excellent compatibility with commercial microarrays.

Finally, we developed the dual-functional magnetic nanochains integrated microfluidic biochip (MiChip) for cancer biomarkers and bacteria analysis. We have shown that because the bioconjugated magnetic nanochains can serve as both nanoscale stir bars to promote rapid liquid mixing and as capturing agents for specific bioseparation, the MiChip eliminates both liquid mixers and surface-anchored sensing elements that are built-in components commonly found in conventional microchips. Thereby, the MiChip features a simple planar design consisting of flat channels free of any target-specific components and becomes a universally applicable platform for a wide spectrum of targets. The MiChip assay allowed rapid (8 min), parallel analysis of small volumes ($\sim 1 \mu\text{l}$) of body fluid specimens, achieving sensitively and selectively quantification and profiling of cancer protein markers in serum samples from 20 cancer patients and specific bacteria in human saliva.

In summary, we successfully developed a novel class of dual-functional magnetic

nanochains based on dopamine self-polymerization strategy. The as prepared magnetic nanochains can undergo synchronized rotation in response to a spinning magnetic field and support easy surface functionalization, which makes them suitable for accelerate liquid mixing and bioseparation in ultra-small volumes. We have successfully demonstrated how to use the magnetic nanochains to address a long-standing challenge, limited diffusion, in DNA and protein microarray assay. Our results have clearly shown that the use of magnetic nanochains led to improved kinetics, increased sensitivities, and reduced signal variation in both of DNA and protein microarray analysis. The magnetic nanochains assisted microarray assays are simple, low cost and compatibility with any commercial microarrays. We further functionalized the magnetic nanochains with specific antibodies for target bioseparation, coupled with SERS probes for signal transduction and integrated them with microfluidic biochip (MiChip) for multiplexing detection of cancer biomarkers and bacteria. In our proof-of-concept studies, we have shown that the MiChip assay delivers ultrasensitive detection of a panel of cancer protein biomarkers and bacterial species in 1 μ l of body fluids within 8 min. The MiChip becomes a generic consumable part for a wide spectrum of analytes, with the magnetic control and Raman detection systems built off-chip for long-term use. This design not only effectively addresses fabricating and translational complexity of the chips with built-in components, overcomes common operational problems associated with complex channel designs such as clogging and poor reproducibility, also helps to

reduce the assay cost.

6.2 Future Research

The complementary advantageous attributes of magnetic nanochains and SERS nanoprobe endow our MiChip platform with great versatility and flexibility for liquid bioassay. In this thesis, we have demonstrated the general utility and robustness of the MiChip platform by rapid, accurate, sensitive, and multiplexed cancer biomarkers detection in clinical serum samples and bacterial detection in human saliva. What is more, the MiChip platform offers versatile opportunities to further extend its use for a broader range of biomarker targets and improve its sensitivity and multiplexing capacity.

The future work will be proposed in the following aspects:

- 1) Improve sensitivity and multiplexing capacity. The current MiChip assay, using SERS nanoprobe for quantitative detection, also offer the flexibility to further improve its sensitivity and multiplexing capacity. The systematically optimized plasmonic nanostructures, such as built-in nanogaps (1-2 nm), can further improve the Raman enhancement efficiency and thus the sensitivity of the MiChip assay¹⁹²⁻¹⁹⁴. Furthermore, the sharp Raman peaks (full width at half maximum, FWHM= 1-2 nm) defined by molecular signatures of the tags makes it possible to further expand the library of

nanoprobes by encoding the plasmonic substrates with molecular tags of different chemical structures.

- 2) Extend MiChip for a broader range of biomarker targets. Taking into account the chemical flexibility to immobilize a diverse type of protein, nucleic acid, and small molecular affinity ligands on the Magchains and SERS probes, we anticipate applications of the McChip assay for protein profiling and genotyping in a wide spectrum of subcellular (*i.e.*, exosomes)^{199, 200} and cellular targets²⁰¹. The rapid, multiplexing signal readout of Raman spectroscopy is of considerable interest for real-time monitoring of interconnected targets, for example, the correlated protein markers in a signaling pathway.
- 3) Our final goal is to develop a fully automated system for magnetic nanochains integrated microchip (MiChip) bioassay.^{202, 203} The assay platform built upon the combinational use of magnetic nanochains and surface-enhanced Raman scattering (SERS) nanoprobes. All the process, including microfluidics manipulating, electromagnet actuation, stage positioning and Raman signal collection and analysis, will be executed with a custom-written LabVIEW program on the computer. Once a raw sample is loaded on the microfluidic chip, the automated system will sequentially perform the processes of sample injection, mixing, targets recognition and separation, and final detection with

minimal intervention from the operator. This platform allows real-time, high-throughput and multiplexed analysis while consuming microliter volume of samples.

- 4) In spite of the promising features, challenges remain in the commercialization of the invented technologies. In actual application, quality control is the primary concern for practitioner. We need to further optimize the preparation of magnetic nanochains and SERS probes such that their properties are consistent and well-controlled. Another challenging in the commercialization may be about the specificity of our products in complex and unprocessed clinical samples. To realize better specificity, we need to further optimize the magnetic nanochains with non-fouling surfaces to reduce non-specific absorption. Mass production of microfluidic chips, magnetic nanochains, and SERS probes is the other challenging in the commercialization. For mass production of the chips, PDMS will be changed to cyclic olefin copolymer (COC) or polypropylene (PP) and standard industrial processes such as injection molding will be adopted instead. In the future, we will scale-up the production of magnetic naochains from milligram to gram level.

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