学校编码：10384　　分类号_____密级_____
学号：20520090153305　　UDC_____

厦门大学

博　士　学　位　论　文

液滴微流控技术及其在化学生物学中的应用

Droplet Microfluidics and Its Application in Chemical Biology

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论文提交日期：2012 年　月

论文答辩时间：2012 年　月

学位授予日期：2012 年　月

答辩委员会主席：_____

评　　阅　　人：_____

2012 年 6 月

Droplet Microfluidics and Its Application in Chemical Biology

A Thesis Presented

by

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SUBMITTED TO THE GRADUATED SCHOOL OF XIAMEN UNIVERSITY

FOR THE DEGREE OF DOCTOR OF SCIENCE

JUNE, 2012

Department of Chemical Biology, Xiamen University

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摘 要

液滴微流控是在微流控芯片上发展起来的一种操纵微小体积液体的全新技术。近年来，随着液滴操控技术的发展与成熟，微流控液滴系统已经成为一个在微观尺度上进行化学和生物学研究的重要平台，并成功应用到蛋白结晶、酶分析、化学合成、单分子/单细胞研究等分子与细胞生物学及分析化学研究领域中。在传统的液滴微流控技术基础上，本文将“油包水”改成“油包琼脂糖”，并提出了一种琼脂糖液滴微流控技术。以微流控芯片上琼脂糖液滴的生成和优化，及其在化学生物学中的应用为目标，探索了微流控芯片的加工，构筑了芯片的基本元件——微阀和微泵，摸索了琼脂糖液滴的产生条件，在传统油包水液滴PCR（聚合酶链式反应）基础上提出了一种琼脂糖液滴PCR的微流控技术，并将其应用于单分子/单细胞检测和核酸适体的筛选。论文工作主要分为以下几个方面：

一、 PMMA/PDMS/PMMA微阀和微泵

为了实现对芯片内流体的精确操控，论文设计一种基于PMMA/PDMS材料的液流驱动系统的芯片微泵。该微泵是由三个微阀串接而成，通过控制微阀中PDMS薄膜的振动频率、PMMA气室体积和气压，可以得到在nL-μL/s范围内精确的可控流速。利用该微泵发展了一种基于微流控芯片的核酸萃取技术，即通过控制芯片中磁珠表面的电荷来实现人全血中核酸的分离和纯化。DNA的萃取效率约为25%，在260 nm和280 nm处紫外吸收的比值为1.72。该微泵加工简单、成本低廉，将有望与便携式、一次性使用的微流控芯片装置实现集成。

二、琼脂糖液滴微流控技术

为解决传统液滴 PCR 效率低且同时保持 DNA 在液滴内的单克隆性，本文在液滴 PCR 体系中引入超低凝胶点的琼脂糖，提出了一种琼脂糖液滴 PCR 的微流控技术。该琼脂糖被用作水相，在普通的流聚焦微流控芯片上能高通量地产生大小均匀的油包琼脂糖液滴，且通过改变芯片通道尺寸和油水两相的流速来实现琼脂糖液滴尺寸的调控。通过表征琼脂糖的物理性质，结果表明这种油包琼脂糖液滴/微球在尺寸上具有良好的单分散性、可控性和热稳定性。

本文以Lambda DNA为例，研究了基于琼脂糖液滴微流控技术的单分子PCR。通过共价偶联的方式将PCR的一条引物偶联到琼脂糖大分子上，使其能将扩增产

物牵制在琼脂糖微球内部不扩散且不影响PCR正常扩增。利用荧光显微镜和流式细胞仪对单分子PCR扩增后的微球进行分析，结果表明：随着每个液滴中平均模板数的增加，荧光微球数目也相应的增加，且符合泊松分布趋势。同时，实时荧光定量PCR结果表明琼脂糖液滴PCR效率可达到80%以上。此方法具有高PCR扩增效率，将在基因测序、肿瘤细胞早期诊断领域具有广泛的应用前景。

三、 琼脂糖液滴微流控技术用于高背景下痕量致病菌的检测

为了解决传统致病菌检测方法灵敏度低且工作量大的问题，本文在琼脂糖液滴单分子PCR技术的基础上发展了一种能在高浓度的非致病菌背景下定量灵敏检测痕量致病菌的方法。用红色和绿色的两种荧光染料分别标记致病菌*E.coli* O157和正常细菌*E.coli* K12。通过提高每个琼脂糖液滴中正常细菌K12的数量，逐级减少致病菌O157的数量，从而降低需要处理的液滴数目。液滴单细胞PCR后，通过在流式细胞仪上测定红色荧光微球和绿色荧光微球的比例来获得*E.coli* O157与*E.coli* K12的比例，检测限达到了$1/10^5$。灵敏度和检测限还能通过增加所分析的液滴数目实现进一步的降低。该方法具有高通量、高灵敏等优点，还将在单细胞表达分析、突变基因检测和癌症的早期诊断等方面有广泛的应用。

四、 琼脂糖液滴微流控技术用于核酸适体的筛选

传统的配体指数富集系统进化技术（SELEX）筛选靶标的核酸适体的整个过程耗时、费力、低效且昂贵。为了解决这些问题，本文采用琼脂糖液滴单分子PCR的微流控技术建立了高效筛选核酸适体的新方法。将癌症标志物SHP2蛋白的单链DNA预富集库按照泊松分布稀释并包裹于独立的均一的琼脂糖液滴中，通过在微流控芯片上的液滴PCR，产生一定比例的包裹单一DNA序列的琼脂糖微球。利用高通量的荧光流式细胞术依次测定液滴中的序列与靶标Shp2蛋白的结合能力，具有高特异性和低平衡解离常数K_d的DNA序列作为靶标Shp2蛋白的核酸适体，并且获得的核酸适体可以直接应用于后续研究。通过该方法，本研究获得了可以选择性识别SHP2蛋白的核酸适体，且核酸适体与SHP2蛋白结合的平衡解离常数K_d为24.9 nM。与传统SELEX的测序-化学合成-结合能力测试的流程相比，本研究建立的方法避免了大量的DNA克隆、测序，避免了昂贵、费时的大量候选DNA序列的合成过程。琼脂糖液滴微流控技术具有高效、廉价等优点，可以广泛应用于分子进化技术，例如mRNA展示，噬菌体展示等。

关键词：微流控液滴，微泵，琼脂糖液滴微流控，致病菌检测，核酸适体

Abstract

Microfluidic droplet is a novel technology based on microfluidic chip that can manipulate ultra-small volume of sample. In recent years, research in droplet-based microfluidics has made great progress. Various techniques concerning the generation and manipulation of droplets have been developed. Droplet based microfluidics systems has become an important platform for microscale chemical and biological researches, including protein crystallography, enzyme analysis, chemical synthesis, single molecule/cell analysis, and so on. In this dissertation, we propose an agarose emulsion droplet microfluidic method by employing ultra-low gelling agarose as dispersed phase based on conventional droplet microfluidics. In order to apply agarose droplet in chemical biology, we have explored the fabrication of microfluidic chip, constructed the basic components of microfluidic chip: valve and pump, and also optimized the generation condition of agarose droplet. Combined with the conventional droplet polymerase chain reaction (PCR), we developed an agarose emulsion droplet microfluidic method for uniform emulsion PCR (ePCR) which was used for single molecule/cell detection and aptamer selection. The main work are listed as follows:

1. PMMA/PDMS/PMMA valve and pump

In order to precisely control the fluid in microchannel, here we presented the design, fabrication, characterization and application of pneumatic microvalves and micropumps based on PMMA (polymethyl-methacrylate). A PMMA diaphragm pump can be assembled by simply connecting three valves in series. A stable and controlled pumping rate ranging from nL to μL per second can be attained by regulating the valve chamber volume, pneumatic pressure, and valve activation time. The PMMA based valves and pumps were further tested in a disposable automatic nucleic acid extraction microchip to extract DNA from human whole blood. The DNA extraction efficiency was about 25% and the 260 nm/280 nm UV absorption ratio for extracted DNA was 1.72. Possessing the advantages of inexpensive, facile

fabrication, and robust, easy integration, the PMMA valve and pump will find their wide applications for fluidic manipulation in portable and disposable microfluidic devices.

2. Agarose droplet microfluidics

To maintain monoclonality of the droplets during downstream processing, and eliminate the disadvantages of traditional ePCR, such as low PCR efficiency, we introduced an agarose emulsion microfluidic method. Monodisperse droplets were produced by injecting ultra-low gelling agarose solution as aqueous phase on a simple microfluidic chip. The diameter of agarose droplets can be easily controlled by adjusting the dimension of chip channel and flow rate of oil and aqueous phase. The physical properties of agarose were characterized，and the experiment results showed a good thermal stability of agarose emulsion and a good mechanical strength of agarose microbeads.

Using lambda DNA as the template, single molecule PCR was explored based on agarose droplet microfluidics. PCR forward primers were covalently coupled to the agarose macromolecules, which can be captured in agarose microbeads during the amplicons generation and avoid low PCR efficiency. These agarose microbeads were then analyzed by fluorescence microscopy or fluorescence activated cell sorter (FACS). The results showed that the number of fluorescent microbeads increased as the mean template number of droplet increasing, and the percentage of microbeads carrying PCR product was in good agreement with the theoretical value predicted by Poisson distribution. Quantitative PCR (Q-PCR) analysis indicated that PCR efficiency in emulsion agarose droplet was higher than 80%. Thus, a high throughput, highly effient single molecule-amplificaiton method was established, which will have wide applications in mutant gene detection, gene sequencing, and early diagnosis of cancer disease.

3. Agarose droplet microfluidic approach for high-sensitive rare pathogenic bacteria detection

Conventional detection methods for Escherichia coli O157:H7 (*E.coli* O157:H7), such as culture-based methods, are time and reagent-consuming,

low sensitive and labor intensive. Herein, we developed an approach that combined the agarose droplets microfluidics, digital PCR, with high-throughput flow cytometry technique to allow the highly sensitive detection of pathogenic bacteria in a quantitative manner from high background of nonpathogenic bacteria. The agarose emulsion droplet microfluidic technology employed agarose with low melting and low gelling temperature, which was coupled with PCR reverse primer using Schiff-base reaction. *E.coli* K12 and *E.coli* O157:H7 were compartmentalized in droplets which varied the O157 cell density from 0.5 cpd to 0.00005 cpd and kept the K12 cell density as 50 cpd. Each droplet contained two kinds of fluorescein-labeled forward primers, one specific for the *E.coli* O157:H7 and the other for the *E.coli* K12, which generated red and green fluorescent signals respectively. After PCR, the droplets were then cooled and converted to microbeads carrying the amplified products. The ratio of *E.coli* O157:H7 to *E.coli* K12 was determined by counting the ratio of red to green beads with flow cytometric analysis. The detection limit of this method was $1/10^5$. The method was sensitive and quantitative. Meanwhile the sensitivity can be higher by increasing the number of the droplets analyzed. Moreover, agarose droplet microfluidics will have potential applications in mutant gene detection, gene sequencing and the early diagnosis of cancer disease.

4. Agarose droplet microfluidic approach for molecular evolution of aptamers

Traditional SELEX process is time-consuming, labor-intensive, inefficient, and expensive. To address these problems, we developed a novel method for efficiently screening affinitive ligands (aptamers) from a complex single stranded DNA (ssDNA) library by employing single-molecule emulsion PCR based on the agarose droplet microfluidic technology. Statistically diluted ssDNA of the pre-enriched library evolved through conventional SELEX against cancer biomarker SHP2 protein was encapsulated into individual uniform agarose droplets for droplet PCR to generate clonal agarose beads. The binding capacity of amplified ssDNA from each clonal bead was screened via high throughput fluorescence cytometry. DNA clones with high binding capacity and low K_d were chosen as the aptamer and could be directly

used for downstream biomedical applications. We have identified a ssDNA aptamer that selectively recognizes SHP2 with a K_d of 24.9 nM. Compared to a conventional sequencing-chemical-synthesis-screening work flow, our approach avoids large-scale DNA sequencing and expensive, time-consuming DNA synthesis of large populations of DNA candidates. Thus the agarose droplet microfluidic approach is highly efficient and cost-effective for molecular evolution and will have wide applications in molecular evolution technologies, including mRNA display, phage display, and so on.

Keywords: Droplet microfluidics; micropump; Agarose droplet microfluidics; Pathogenic bacteria detection; Aptamer

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