

Article

Integrated Microfluidic Chip Enabling Preparation and Immobilization of Cell-Laden Microspheres, and Microsphere-Based Cell Culture and Analysis

Qiongyao Mou ^{1,2,3}, Peiyi Zhang ³, Daijing Li ³, Qiong Wang ⁴ and Jun Yang ^{3,*}

¹ Key Laboratory of Acupuncture-Moxibustion and Tuina Intelligent Equipment of Chongqing Administration of Traditional Chinese Medicine, Chongqing Three Gorges Medical College, Chongqing 404120, China; 20240001@cqtgmc.edu.cn

² Research Center of Oral Materials and Technology, Department of Medical Technology, Chongqing Three Gorges Medical College, Chongqing 404120, China

³ Key Laboratory of Biorheological Science and Technology, Ministry of Education and Bioengineering College, Chongqing University, Chongqing 400044, China; 202019131122@cqu.edu.cn (P.Z.); 13438416872@163.com (D.L.)

⁴ Department of Basic Medicine, Chongqing Medical and Pharmaceutical College, Chongqing 401331, China; wangqiong@cqu.edu.cn

* Correspondence: bioyangjun@cqu.edu.cn

Abstract

Microfluidics-based preparation methods for cell-laden hydrogel microspheres are well-suited for large-scale comparative analysis of single or few cells. However, in existing studies, the preparation of cell-laden hydrogel microspheres and the cell culture process are typically separated, requiring the fabricated microspheres to be eluted and transferred from the preparation device to cell culture dishes or plates for cultivation. This transfer process can easily compromise sterility, while conventional cell culture methods consume more reagents and cause microsphere stacking, hindering single-cell observation and analysis. To address these issues, this paper presents an integrated microfluidic chip that sequentially enables droplet generation with cell encapsulation, gel droplet solidification, hydrogel microsphere trapping, and microsphere-based cell culture and analysis, facilitating the cultivation and observation of single or small numbers of cells. Integrating cell-laden microsphere preparation and 3D cell culture within a sealed chip structure reduces contamination risks associated with cell transfer, enables automation of multiple cell analysis workflows, and minimizes reagent and sample consumption. Using polydimethylsiloxane (PDMS) with good gas permeability and processability as the chip material, biocompatible fluorinated oil was selected as the oil phase for microsphere preparation. A mild sodium alginate-calcium ion gelation system was employed, where calcium ions were released under acidic conditions after droplet generation to trigger solidification, yielding uniform hydrogel microspheres. Under optimized conditions, the single-cell encapsulation efficiency for test samples of human myeloid leukemia cells (K562) was 33.8% $\pm$ 1.8%, with a size uniformity coefficient of variation (CV) reaching 3.85%. Cells encapsulated within hydrogel microspheres were cultured in 286 on-chip independent cell culture chambers, achieving >95% viability after 24 h.

Keywords: microfluidics; droplet; gel microsphere; cell encapsulation; single-cell culture

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1. Introduction

Cells are the fundamental units of life, and their various behaviors are closely related to physiological and pathological processes such as growth and development, disease progression, repair, and aging of organisms. Micron-scale structures on microfluidic chips enable precise manipulation of cells while offering advantages including low sample consumption, rapid response, and excellent biocompatibility. These devices have found widespread applications in cell culture and analysis [1], cell fusion [2], cell electroporation [3], drug screening [4], and other fields. Since the living environment profoundly influences cellular activities, simulating cellular microenvironments *in vitro* to study cell behavior has become a hotspot in biological research. Most related studies rely on simultaneous culture of large cell populations, detecting the entire cell group to obtain statistical results. However, average population responses often mask individual differences between cells—for instance, cells of the same genotype in identical environments may differ in morphology, functional expression, proliferation, differentiation, and migration [5]. Such cellular heterogeneity affects biological processes including cell differentiation, embryonic development, and disease evolution [6]. To fully understand cellular diversity and heterogeneity, comprehensive analysis at the single-cell level is essential [7]. Single-cell culture enables real-time monitoring of individual cell behaviors and generates sufficient clonal cells and their derivatives [8], avoiding the influence of neighboring cells and their metabolic byproducts (amino acids, sugars, signaling molecules, fatty acids, etc.) encountered in population culture. Traditional single-cell analysis equipment is expensive, with complex and time-consuming procedures, significantly limiting its application in studying cellular heterogeneity [9]. Microfluidic devices leverage their fine manipulation capabilities to simulate *in vivo* cellular growth environments for studying interactions between individual cells and the extracellular matrix, as well as cell–cell interactions. These platforms are no longer limited to planar culture operations and can integrate functions such as real-time monitoring and analysis, multiple physicochemical gradients, cell capture, and sorting [10].

In microfluidic single-cell analysis, individual cells are typically constrained using hydrodynamic devices, microwells, or microstructure arrays [11–13]. Beyond direct cell manipulation, cell encapsulation represents another viable approach [14–16]. Cells are enclosed within semi-permeable encapsulation layers featuring porous structures, allowing free passage of small molecules such as oxygen, nutrients, and metabolites while blocking larger substances including immune cells, antibodies, and enzymes. This provides protection for the encapsulated cells while maintaining their viability [17,18]. The encapsulation layer can also serve as an extracellular matrix substitute, forming three-dimensional scaffolds that provide structural support and resistance against external physical stress [19,20]. Encapsulation structures can be categorized into bulk encapsulation [21] containing large numbers of cells and microcapsule encapsulation [22] of single or multiple cells. Excessive encapsulation volume hinders mass transport and exchange, leading to the gradual replacement of bulk encapsulation by microcapsules in single-cell research. Examples include microcapsule structures based on natural polymer materials such as polyethylene glycol, chitosan, sodium alginate, agarose, hyaluronic acid, and collagen.

Traditional methods can produce cell-laden droplets through extrusion or similar processes, which are then solidified to form cell-loaded microspheres. The encapsulation layer thickness has been reduced from hundreds of micrometers or even several millimeters in bulk encapsulation to less than 100 μm , significantly improving the transport efficiency of oxygen, nutrients, metabolites, and other substances. However, these methods suffer from low efficiency, poor size uniformity, and highly unstable cell encapsulation rates. Droplet microfluidics technology leverages the emulsification effect between immiscible liquids (such as oil and water) to prepare droplets containing single cells, which

are then crosslinked and solidified to obtain microspheres. This approach has achieved efficient encapsulation of various animal, plant, and microbial cells, and has been widely applied in injectable cell therapy, bioreactor-based cell expansion and differentiation, and high-throughput drug testing and analysis [23–26]. Droplet microfluidic devices can generate large quantities of monodisperse droplets in a short time and control their shape, size, and contents by adjusting parameters. Under such random encapsulation conditions, the single-cell encapsulation efficiency in droplets follows a Poisson distribution:

$$P(\lambda, k) = \lambda^k e^{\frac{-\lambda}{k!}}, \quad (1)$$

where k represents the number of cells within a droplet, and λ represents the average number of cells per droplet. According to Equation (1), the theoretical maximum percentage of droplets containing single cells is 37% [27]. By adjusting cell concentration, suspension medium density, and fluid flow rates [28]; optimizing channel structures [29]; and combining with sorting methods [30], it is possible to overcome the Poisson distribution limitation and achieve higher-efficiency single-cell encapsulation.

In existing research on cell-laden gel microspheres, the microsphere fabrication and cell culture stages are largely separate [31]. The prepared cell-laden gel microspheres need to be transferred to culture dishes or well plates for cell culture. For example, Seeto et al. used a custom-designed T-junction to generate droplets, rapidly crosslinked them with high-power visible light, and then transferred the resulting gel microspheres to collagen-coated well plates for long-term culture [32]. These studies must be conducted in a sterile environment to avoid cell contamination. Meanwhile, traditional culture devices consume relatively large amounts of reagents, and the stacking of large quantities of gel microspheres can easily lead to gel deformation or fusion, affecting cell function and experimental observation. Moreover, microspheres are randomly dispersed in culture dishes or well plates and are susceptible to disturbances from operations such as medium changes, making accurate positioning impossible and hindering continuous observation and analysis.

Microstructure arrays are commonly employed in microfluidic chips for trapping applications, enabling precise separation of cells and microparticles as well as large-scale manipulation. To address the limitations of existing studies based on cell-laden microspheres, we integrate arrayed trapping microstructures with microsphere-based cell encapsulation to achieve precise single-cell trapping, culture, and real-time monitoring and analysis. This study presents a workflow-integrated and modularized design on a single microfluidic chip, enabling sequential generation and solidification of cell-laden droplets, microsphere trapping, and gel microsphere-based cell culture and analysis. Under optimized conditions, the chip generates monodisperse gel microspheres (average diameter 95 μm , CV = 3.85%) with a single-cell encapsulation efficiency of 33.8%. On-chip culture of K562 cells further demonstrates excellent biocompatibility, achieving >95% cell viability after 24 h. The integrated and enclosed 3D cell culture chip system effectively eliminates contamination risks associated with cell transfer, enhances the degree of automation in analysis, and reduces reagent and sample consumption.

2. Experimental Section

2.1. Materials

Photoresist SU-8 3050 was purchased from MicroChem Corporation (Newton, MA, USA). Polydimethylsiloxane (PDMS) and curing agent were obtained from Dow Corning Corporation (Midland, MI, USA). Calcium chloride was sourced from Chuandong Chemical Group (Chongqing, China). Sodium alginate was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Disodium ethylenediaminetetraacetate (EDTA-

2Na), perfluorooctanol (PFO), and sodium hydroxide were obtained from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Acetic acid (HAc) was purchased from Saan Chemical Technology Co., Ltd. (Shanghai, China). NOVEC HFE 7100 was sourced from 3M China Co., Ltd. (Shanghai, China). Trypan blue was obtained from Shuopu Biotechnology Co., Ltd. (Guangzhou, Guangdong, China). Nile blue was purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Aquapel was sourced from PPG Industries, Inc. (Pittsburgh, PA, USA). RPMI Medium 1640 was purchased from Thermo Fisher Biochemical Products (Beijing) Co., Ltd. (Beijing, China). Fetal bovine serum was obtained from Nuobo Biological Products Co., Ltd. (Hangzhou, Zhejiang, China). Penicillin-streptomycin solution was purchased from Saiguo Biotechnology Co., Ltd. (Guangzhou, Guangdong, China). The K562 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Serial: SCSP-5054, Identifier: CSTR:19375.09.3101HUM-SCSP5054, Shanghai, China).

2.2. Chip Design and Fabrication

The microfluidic chip (Figure 1) mainly comprises two functional modules: a module for preparing cell-laden hydrogel microspheres and a module for microsphere capturing and subsequent cell culture. Microsphere fabrication is based on droplet generation methods, commonly including microchannel structures such as coaxial capillaries, flow-focusing devices, and T-junctions. Among these, coaxial capillaries require demanding fabrication processes and are relatively expensive, while T-junctions offer limited flexibility in fluid control. Therefore, this study employed a cross-flow focusing structure to prepare cell-laden hydrogel microspheres. Cells were suspended in the hydrogel precursor solution as the dispersed phase, which interacted with the continuous phase to form cell-encapsulating droplets. To ensure uniformity of the encapsulating hydrogel layer, good biocompatibility, and supportive and protective functions, a sodium alginate–calcium ion gel system was selected. Calcium ions were pre-chelated within the sodium alginate and subsequently released under acidic conditions after droplet generation to trigger droplet solidification, yielding hydrogel microspheres.

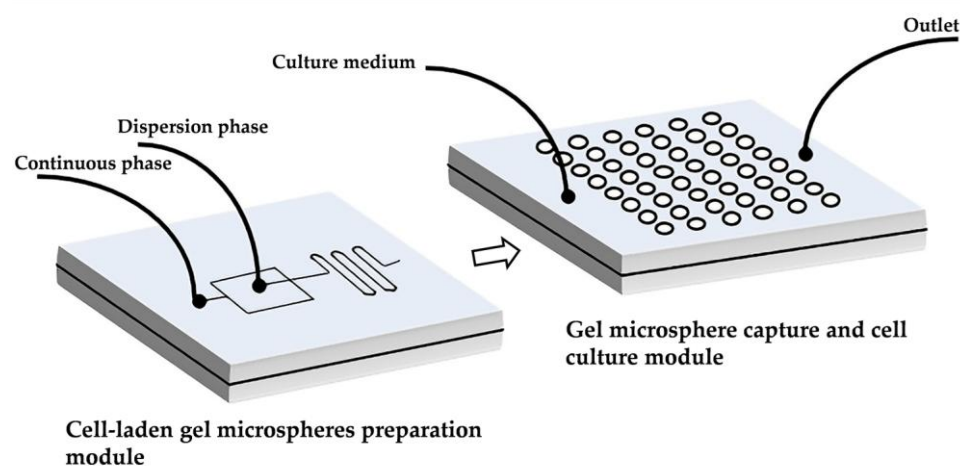


Figure 1. Schematic of the integrated microfluidic chip design.

The size and structure of the hydrogel microspheres are governed by the droplet generation and gel polymerization processes, influenced by parameters including channel width, flow rates, and the flow rate ratio between the two phases. Compared with mineral oils used in conventional approaches, fluorinated oils offer superior biocompatibility; therefore, fluorocarbon oil HFE-7100 was employed as the continuous phase. This enables spontaneous demulsification of droplets following gel solidification, streamlining the

production workflow while enhancing the viability of encapsulated cells. The solidification channel geometry modulates droplet solidification by affecting the solidification duration. Upon formation, the cell-laden hydrogel microspheres are transported to the downstream capturing and culture module for arrayed single-microsphere immobilization and in situ cell culture. To generate thinner hydrogel layers conducive to cell growth, encapsulation is typically conducted at high flow rates; however, this introduces challenges including heightened shear stresses on cells and exposure to acidic conditions that may compromise cell viability, alongside the technical difficulty of precisely immobilizing individual microspheres. To address these issues, a wash phase is introduced at the inlet of the capturing and culture module to enable buffer exchange and pH neutralization during microsphere preparation, thereby preventing viability loss from prolonged exposure to acidic oil. This architecture also supports continuous perfusion of culture medium throughout subsequent operations.

The chip structure was designed using AutoCAD 2024 software (Autodesk, San Francisco, CA, USA) and subsequently fabricated into photomasks for microfabrication. Polydimethylsiloxane (PDMS), selected for its ease of microfabrication; low cost; and excellent elasticity, biocompatibility, and gas permeability, was employed as the chip material. The microfabrication process involved first constructing photoresist structures on silicon wafers via photolithography to serve as molds, followed by PDMS microfluidic chip fabrication using soft lithography. Specifically, 3-inch silicon wafers were immersed in absolute ethanol and subjected to plasma cleaning for 3 min, subsequently rinsed with deionized water, blow-dried with nitrogen, and baked at 90 °C for 5 min to complete wafer cleaning. Approximately 3 mL of SU-8 3050 negative photoresist was dispensed onto the silicon wafer, spin-coated at 500 rpm for 10 s followed by 3000 rpm for 30 s, and soft-baked at 95 °C for 15 min. Following UV exposure, the wafers were baked at 65 °C for 1 min and 95 °C for 5 min post-exposure, cooled to room temperature, developed for 5–8 min, and hard-baked at 150 °C for 60 min to ensure complete solvent evaporation and enhanced structural stability. PDMS base and curing agent were mixed thoroughly in a 10:1 ratio, degassed under vacuum to remove air bubbles, poured into acrylic molds containing the silicon wafer templates, and cured at 65 °C for 30 min. The cured PDMS was peeled off, and access holes were punched at designated locations. A flat PDMS slab was prepared as the base substrate using a bare silicon wafer. Both the structured PDMS layer and the flat substrate were subjected to oxygen plasma treatment for 15 s and immediately bonded together. Polytetrafluoroethylene (PTFE) tubing was inserted into the pre-punched holes, and the interfaces were sealed with uncured PDMS mixture to obtain the completed microfluidic chip. Glass-based microfluidic chips were fabricated using a similar approach, with Aquapel hydrophobic agent (PPG Industries, Inc., Pittsburgh, PA, USA) pumped through the channels at 20 µL/h using a syringe pump (LSP10-1B, Baoding Longer Precision Pump Co., Ltd., Baoding, China) for surface hydrophobization.

2.3. Cell Viability Assay

Cell-laden hydrogel microspheres permit the passage of small molecules, thereby enabling viability assessment of encapsulated cells via trypan blue staining. For detection, the microsphere suspension was mixed with 0.4% trypan blue solution at a 9:1 ratio. After incubation for 2–3 min, the samples were examined under a microscope to enumerate live and dead cells. Cells with compromised membrane integrity were considered dead, appearing pale blue and slightly enlarged, whereas live cells remained colorless and transparent with normal morphology. Only cells within the hydrogel microspheres were counted, with a sample size exceeding 100.

2.4. Characterization of Hydrogel Microsphere Size and Uniformity

Hydrogel microspheres are colorless and transparent. To facilitate microscopic observation and accurate size measurement, staining was required. Nile blue was employed to stain the microspheres pale blue by mixing the Nile blue solution with the hydrogel microsphere suspension at a 1:2 ratio. Following 30 s of incubation, the microspheres were fully stained and subsequently observed under an optical microscope with images captured by a camera. Particle size was measured using the image processing software ImageJ (version 1.52a, bundled with 64-bit Java 8, National Institutes of Health, Bethesda, MD, USA), with a sample size exceeding 100 microspheres.

2.5. Measurement of Encapsulation Efficiency

Nile blue solution stains cells blue-black, whereas the hydrogel matrix appears pale blue. The Nile blue solution was mixed with cell-laden hydrogel microsphere suspension at a 1:2 ratio. Following 30 s of incubation for complete staining, the samples were examined under an optical microscope to enumerate empty microspheres, single-cell-laden microspheres, and microspheres containing two or more cells, with a total sample size exceeding 100.

3. Results and Discussion

3.1. Generation of Cell-Laden Hydrogel Microspheres Using Droplet Microfluidic Systems

Droplet size and the mechanical forces involved in droplet formation are critical factors influencing the survival and proliferation of encapsulated cells. As the diameter of hydrogel microspheres decreases, the specific surface area increases, which is more conducive to mass transfer between the encapsulated cells and the external environment, enabling faster absorption of nutrients and oxygen and elimination of metabolic waste, thereby effectively improving cell viability. Studies have shown that when microsphere diameter exceeds 150 μm , cells encapsulated within will undergo hypoxic death [33]. The droplet formation process is primarily governed by fluid shear stress, with droplet size correlating with factors such as microfluidic channel width and flow rate. Generally, narrower channels and higher flow rates generate greater shear forces that cause more cellular damage. Increasing channel dimensions and reducing flow rates can shorten the duration of exposure to harmful environments, effectively enhancing the activity of encapsulated cells; however, this also increases the encapsulation layer thickness, which is detrimental to mass transfer. Therefore, optimal fabrication conditions must be explored. Furthermore, when using an HAc solution prepared with fluorinated oil as the continuous phase and sodium alginate containing chelated calcium ions as the dispersed-phase prepolymer solution, the concentration of HAc also affects the preparation efficacy of the hydrogel microspheres.

During the experiment, HFE-7100 was used to prepare a mixture containing 5% (*v/v*) PFO and a specific concentration of HAc as the continuous phase. Deionized water was used to prepare a 2% (*w/v*) alginate solution and a 0.2 M Ca-EDTA solution. The pH of the Ca-EDTA solution was adjusted to 7 using NaOH solution, and the prepolymer solution was formed by mixing equal volumes of the alginate and Ca-EDTA solutions as the dispersed phase. The dispersed phase and continuous phase were separately injected into the microfluidic chip. The flow rates of both phases were adjusted to ensure that the continuous phase preferentially wetted the channel surface. At the junction of the two channels, a monodisperse water-in-oil single emulsion was formed due to the emulsification effect. The droplets solidified in the downstream channel of the cross-junction. The HAc from the continuous phase diffused into the dispersed phase, releasing the calcium ions

chelated with EDTA. The free calcium ions cross-linked with alginate to induce gelation, thereby solidifying the droplets into hydrogel microspheres.

3.1.1. Effect of Solidification Microchannel Configuration

In the cross-junction microfluidic structure used for hydrogel microsphere preparation, the continuous phase channels were symmetrically distributed in two paths. The total channel length from the inlet to the cross-junction was 2.0 cm, comprising 1.2 cm along the flow direction and 0.4 cm perpendicular to it, while the dispersed phase traveled 0.4 cm from its inlet to the cross-junction. In the preliminary design, the long straight channel utilized for droplet solidification was 2.0 cm in length, with the overall dimensions of the microchannel structural area being 0.8 cm × 3.2 cm (Figure 2A). Under appropriate channel configurations and flow velocities, droplets were observed to form stably at the focusing junction, subsequently passing through the long straight solidification channel and exiting via the chip outlet. However, staining of the collected liquid at the outlet revealed no hydrogel microspheres. When the buffer solution was replaced with either 0.2% (*v/v*) HAc solution or 0.2 M CaCl₂, large gel aggregates formed by the aggregation of numerous microspheres were observed at the chip outlet (Figure 2B).

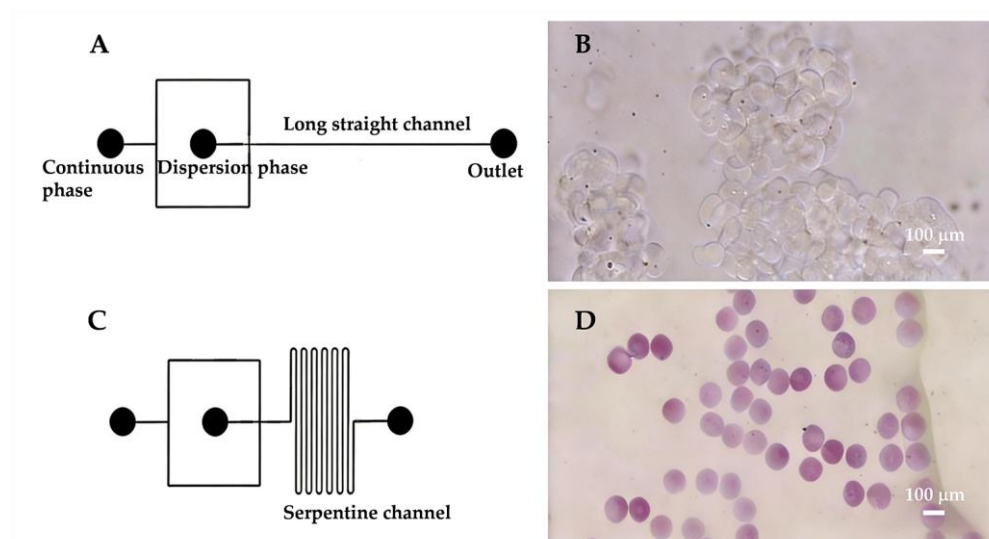


Figure 2. Microsphere preparation efficacy using different solidification microchannel configurations. (A) Long straight solidification channels and (C) serpentine solidification channels, with their corresponding hydrogel microsphere preparation results shown in (B) and (D), respectively.

The formation of gel agglomerates may be attributed to the insufficient length of the curing channel, which hindered the smooth and continuous formation of microspheres. To increase the channel length, the improved design adopted a serpentine configuration with 12 bends, achieving a total length of 12.5 cm and overall dimensions of 1.0 cm × 2.5 cm for this region (Figure 2C). Under identical conditions, stable droplet generation was observed at the focusing structure. After passing through the serpentine curing channel, the droplets were expelled from the chip outlet, where well-formed gel microspheres could be clearly observed, exhibiting uniform size and excellent monodispersity upon staining (Figure 2D). In an acidic environment, chelated calcium ions are released and subsequently react with alginate chains to form calcium alginate gel. Acetic acid (HAc) provides the acidic environment for the droplets, while calcium chloride (CaCl₂) supplies sufficient calcium ions; the complete release of these chelated ions facilitates gel microsphere formation at the outlet. Consequently, the residence time of droplets in the acidic environment influences calcium ion release and, in turn, the droplet gelation process. The

straight curing channel, being both linear and short, allows droplets to flow through rapidly, which is unfavorable for complete curing reactions and results in difficulty forming intact microspheres. In contrast, the longer serpentine curing channel significantly increases droplet residence time, promoting complete calcium ion release and successful gel microsphere formation.

3.1.2. Effects of Microchannel Surface Wettability and Chip Material Selection

The surface hydrophilicity/hydrophobicity of microchannels affects fluid motion and droplet formation. The wetting properties of solid surfaces can be quantitatively analyzed through contact angles. In droplet-based microfluidic chips, the contact angle between the fluid and channel walls determines whether the fluid can flow stably within microchannels to generate various flow patterns. For water-in-oil droplet generation, when the continuous phase completely wets the microchannel while the dispersed phase does not wet or only partially wets the channel walls, the generated droplets can flow stably within the channel; however, when the dispersed phase exhibits strong wetting toward the channel walls, the flow behavior of the liquid phase becomes uncontrollable. When the contact angle between the dispersed phase and channel walls is less than 90° , droplet or filament generation cannot be controlled, whereas when the contact angle exceeds 90° , droplets can be generated stably and flow orderly within the channel [34]. Therefore, following chip fabrication, the channels typically require specific hydrophilic/hydrophobic treatment to enable droplet generation. If employing the commonly used PDMS–glass chip structure, the continuous phase (HFE-7100) completely wets the PDMS channel walls, while sodium alginate droplets exhibit a contact angle of 28.9° with untreated glass surfaces, demonstrating strong hydrophilicity that prevents orderly droplet flow. In contrast, the contact angles of sodium alginate droplets with hydrophobically treated glass surfaces and PDMS surfaces are 135.9° and 115.5° , respectively. This indicates that in selecting chip materials, the PDMS–glass structural model can be abandoned in favor of PDMS–PDMS bonded microfluidic chips, which enable effective droplet preparation without additional channel surface treatment, significantly reducing both fabrication complexity and experimental difficulty. K562 cell encapsulation tests were conducted using untreated PDMS microfluidic chips and hydrophobically treated glass–PDMS chips. The PDMS chips successfully generated droplets stably, yielding cell-laden hydrogel microspheres. The preparation performance—including microsphere size, size uniformity, and cell encapsulation efficiency—showed no significant difference compared to hydrophobically treated glass–PDMS chips.

3.1.3. Effect of Channel Dimensions, Flow Rate, and Acetic Acid Concentration on Droplet Formation

In microfluidic chips, droplet generation primarily arises from the emulsification between aqueous and oil phases, governed by channel properties (channel geometry, dimensions, and wettability), fluid properties (viscosity, contact angle with channel walls, and interfacial tension between phases), and operating parameters (flow rate, external magnetic/electric fields, and temperature). The characteristics of two-phase flow depend on the balance between inertial, viscous, and surface tension forces, which can be analyzed using the Capillary number Ca (ratio of viscous forces to interfacial tension), Reynolds number Re (ratio of inertial to viscous forces), and Weber number We (ratio of inertial effects to interfacial tension). These factors primarily influence the flow patterns formed after convergence of the two phases, such as dripping, jetting, and tubing flow. In the dripping regime, droplets exhibit uniform size distribution with stable generation frequency and higher monodispersity, typically occurring at relatively low dispersed phase flow rates, whereas jetting occurs when the dispersed phase flow rate is higher. To ensure

cell encapsulation efficiency and post-encapsulation cell viability, it is necessary to investigate and optimize the influence of these physical parameters on droplet formation. During experiments, the dispersed phase flow rate was fixed at 30 $\mu\text{L/h}$, while the continuous phase flow rate was gradually increased in increments to observe and record droplet generation states and flow pattern transitions at various flow rate ratios, with hydrogel microspheres subsequently collected for statistical analysis.

Microfluidic chips with flow-focusing structures featuring channel widths of 30 μm , 45 μm , 60 μm , and 75 μm were tested and analyzed respectively. At low continuous phase flow rates (<200 $\mu\text{L/h}$), the dispersed phase could not be completely sheared and broken into droplets, and the flow remained laminar after convergence of the two phases. The dispersed phase liquid entered the downstream channel of the focusing structure, where acetic acid (concentration 0.2% (v/v)) from the continuous phase diffused into the dispersed phase, causing partial gelation and formation of micro-filaments. The increased viscosity of the dispersed phase resulted in slow movement within the channel, making clogging likely to occur. As the continuous phase flow rate further increased, the flow after convergence exhibited jetting behavior, characterized by elongated necking of droplets and slight gelation reactions at the interface between the two phases. The flow rate range for this jetting regime was very narrow. Upon further increase in continuous phase flow rate, the flow transitioned to dripping flow, which was maintained over a broad range of flow rates. However, as the continuous phase flow rate continued to increase excessively, the excessive pressure caused the continuous phase to surge back into the dispersed phase channel, resulting in complete blockage. When the dispersed phase flow rate was fixed at 30 $\mu\text{L/h}$, the continuous phase flow rate ranges for dripping flow on chips with channel widths of 30 μm , 45 μm , 60 μm , and 75 μm were 270–1100 $\mu\text{L/h}$, 240–1050 $\mu\text{L/h}$, 210–950 $\mu\text{L/h}$, and 180–900 $\mu\text{L/h}$, respectively. As the channel width increased, both the upper and lower limits of the dripping flow regime decreased significantly (Figure 3A).

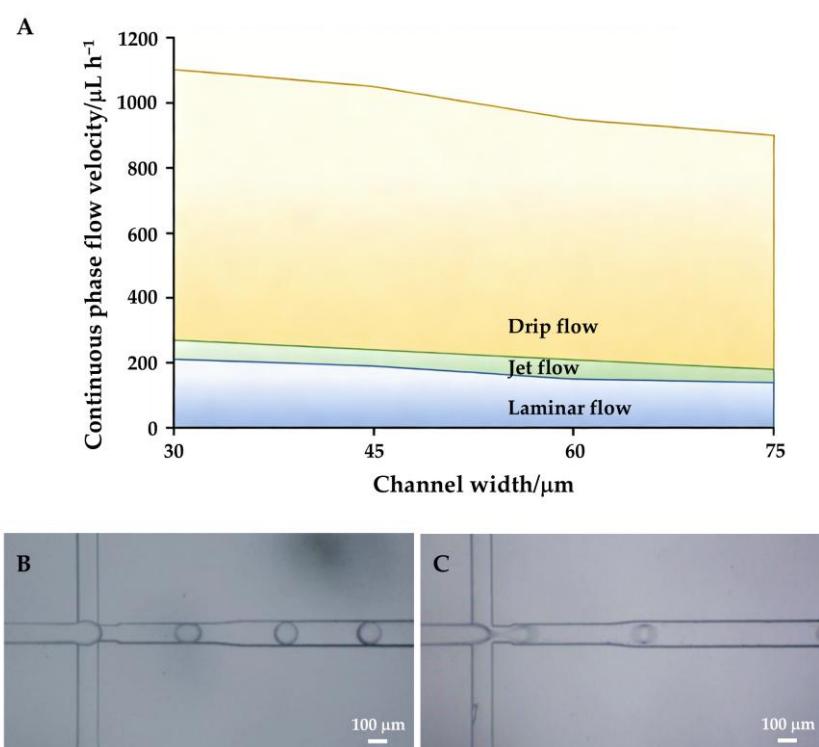


Figure 3. Influence of channel width and flow rate on hydrogel droplet formation. (A) Flow regime maps at various continuous phase flow rates under different channel width conditions at a fixed

dispersed phase flow rate of 30 $\mu\text{L/h}$; effects of low (**B**) and high (**C**) continuous phase flow rates on droplet morphology at a channel width of 75 μm .

Droplet size affects cell encapsulation efficiency and the viability of encapsulated cells and is primarily related to the capillary number (Ca) and channel width. When the dispersed phase flow rate remains constant, an increase in the continuous phase flow rate enhances the shear force exerted on the dispersed phase fluid, reducing the filling time during droplet generation and facilitating droplet breakup. The channel width restricts the lateral expansion of droplets, determining the maximum droplet diameter (approximately equal to the channel width) during stable continuous generation in the dripping regime. With fixed compositions of the continuous and dispersed phases, the fluid viscosity μ and interfacial tension σ between the two phases remain constant, and the continuous phase flow rate then determines the capillary number Ca . Based on the droplet generation range (Figure 3A), the dispersed phase flow rate was set at 30 $\mu\text{L/h}$, and continuous phase flow rates of 350 $\mu\text{L/h}$, 450 $\mu\text{L/h}$, 550 $\mu\text{L/h}$, 650 $\mu\text{L/h}$, 750 $\mu\text{L/h}$, 850 $\mu\text{L/h}$, and 950 $\mu\text{L/h}$ were selected for testing. At a continuous phase flow rate of 350 $\mu\text{L/h}$, the droplet diameter was approximately equal to the width of the solidification channel, with small inter-droplet spacing (Figure 3B). Under otherwise identical conditions, the droplet diameter was regulated by the continuous phase flow rate: as the flow rate increased from 350 $\mu\text{L/h}$ to 650 $\mu\text{L/h}$, the particle size gradually decreased, the inter-droplet spacing increased, and the generation frequency became faster. When the continuous phase flow rate increased beyond 650 $\mu\text{L/h}$ (e.g., 750 $\mu\text{L/h}$, Figure 3C), the diameter of the hydrogel microspheres tended to stabilize. As the continuous phase flow rate increased from 350 $\mu\text{L/h}$ to 950 $\mu\text{L/h}$, the microsphere diameter ranged from 80 μm to 115 μm . On the other hand, the microfluidic channel geometrically constrains the expansion of generated droplets, also determining the microsphere size to a certain extent. Under otherwise identical conditions, smaller channel widths produce smaller microspheres; for every 15 μm decrease in width, the particle diameter in the stable state decreases by approximately 5 μm .

The uniformity of cell-laden hydrogel microsphere size significantly influences the cultivation of encapsulated cells, determining the consistency of the culture environment. Meanwhile, higher microsphere uniformity substantially reduces the design and operational complexity of microsphere capture arrays. The uniformity of hydrogel microspheres is quantified using the coefficient of variation (CV), defined as the ratio of standard deviation to mean, which reflects the degree of dispersion in the data. When the continuous phase HAc concentration was 0.2% (v/v) and the flow rates of the dispersed phase and continuous phase were 600 $\mu\text{L/h}$ and 30 $\mu\text{L/h}$, respectively, the size uniformity of hydrogel microspheres prepared using four different channel widths was evaluated. The resulting particle size uniformity for channel widths of 75 μm , 60 μm , 45 μm , and 30 μm was 3.85%, 3.59%, 3.53%, and 2.13%, respectively, indicating that hydrogel microspheres with high size uniformity can be obtained under the selected experimental conditions.

3.1.4. Effect of Physical Parameters on Cell Encapsulation Efficiency in Cell-Laden Gel Microspheres

Parameters such as microchannel configuration influence the size of droplets and the resulting microspheres, as well as cell encapsulation efficiency. Experimental studies were conducted under various preparation conditions, aiming to minimize microsphere size while maintaining comparable encapsulation efficiency to achieve higher cell viability. In the experiments, appropriate amounts of K562 cells were added to the dispersed phase solution, and the cell encapsulation status and viability of the formed microspheres under different conditions were tested and statistically analyzed. When the HAc concentration in the continuous phase was 0.2% (v/v) and the K562 cell density in the dispersed phase

was approximately 2×10^6 cells/mL, two channel widths that produced microspheres with relatively ideal dimensions were examined: 75 μm and 45 μm . Single-cell encapsulation under different flow rate ratios was investigated. The results from five replicate experiments are shown in Figure 4. When the dispersed phase flow rate was 30 $\mu\text{L/h}$ and the continuous phase flow rate was below 600 $\mu\text{L/h}$, the single-cell encapsulation efficiency of the 45 μm width channel was higher than that of the 75 μm width channel. However, when the dispersed phase flow rate was 30 $\mu\text{L/h}$ and the continuous phase flow rate ranged from 600 $\mu\text{L/h}$ to 800 $\mu\text{L/h}$, the single-cell encapsulation efficiency of the 75 μm width channel was significantly higher than that of the 45 μm width channel. During microsphere formation, the particle size decreased with increasing continuous phase flow rate and stabilized when the continuous phase flow rate exceeded 600 $\mu\text{L/h}$ (Figure 4). Higher continuous phase flow rates imply greater shear forces during cell encapsulation. To minimize microsphere size while reducing fluid shear force and achieving satisfactory single-cell encapsulation, the optimal conditions were determined to be: microchannel width of 75 μm , dispersed phase flow rate of 30 $\mu\text{L/h}$, and continuous phase flow rate of 600 $\mu\text{L/h}$.

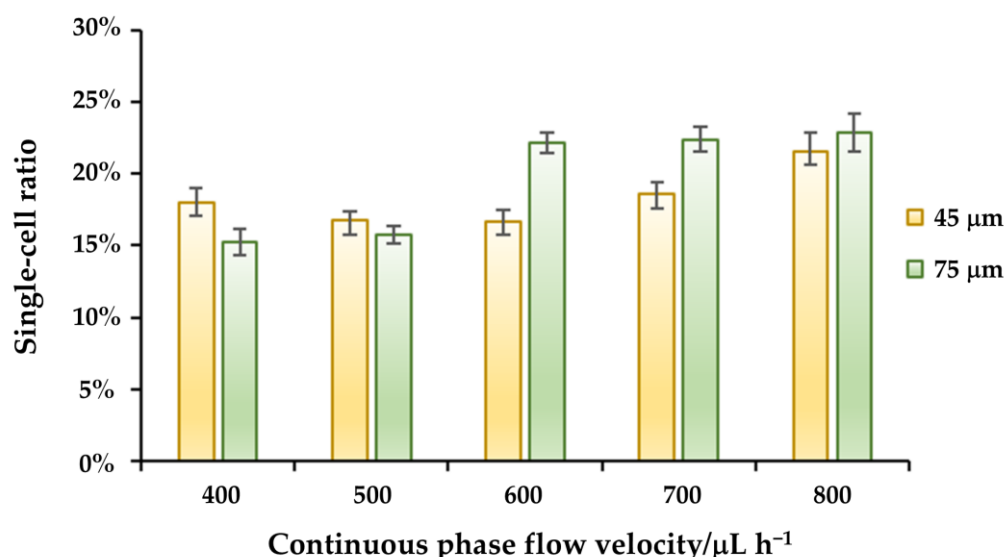


Figure 4. Single-cell encapsulation efficiency of gel microspheres prepared with different channel widths under identical parameters.

3.1.5. Effect of HAc Concentration on the Preparation of Cell-Laden Gel Microspheres

Acetic acid concentration also affects continuous phase viscosity and droplet formation. To investigate the effect of HAc concentration on the preparation of cell-laden gel microspheres in depth, mixed solutions were prepared with HFE-7100 containing 5% (v/v) PFO and varying HAc concentrations of 0.01% (v/v), 0.2% (v/v), 0.3% (v/v), and 0.5% (v/v) as the continuous phase. Fabrication experiments were conducted using a chip with a flow-focusing microchannel width of 75 μm . The continuous and dispersed phase flow rates were fixed at 600 $\mu\text{L/h}$ and 30 $\mu\text{L/h}$, respectively, with appropriate amounts of K562 cells added to the dispersed phase. The droplet pinch-off time refers to the duration required for the continuous phase shear force to overcome the dispersed phase viscosity and interfacial tension, shearing the dispersed phase into droplets. Under a fixed dispersed phase flow rate, increasing the continuous phase flow rate can enhance the continuous phase shear force, effectively shortening the pinch-off time, thereby generating smaller droplets. Elastic stress suppresses the necking process, whereas capillary stress accelerates filament thinning until breakup. When elastic stress dominates, only uniform

filaments or “beads-on-a-string” structures form, whereas capillary stress dominance results in droplet formation. At the flow-focusing junction, HAc in the continuous phase induces partial gelation of the dispersed phase. Higher HAc concentrations lead to greater gelation extent, resulting in more pronounced elastic stress in the dispersed phase and greater resistance to droplet pinching. At the same flow rate ratio, continuous phases with HAc concentrations of 0.1% (*v/v*) and 0.2% (*v/v*) could shear the dispersed phase to generate droplets (Figure 5(A1)). When the HAc concentration increased to 0.3% (*v/v*), the dispersed phase underwent partial gelation and could not be completely sheared and pinched off by the continuous phase, forming typical “beads-on-a-string” structures (Figure 5(A2)). When the HAc concentration further increased to 0.5% (*v/v*), long filaments formed (Figure 5(A3)).

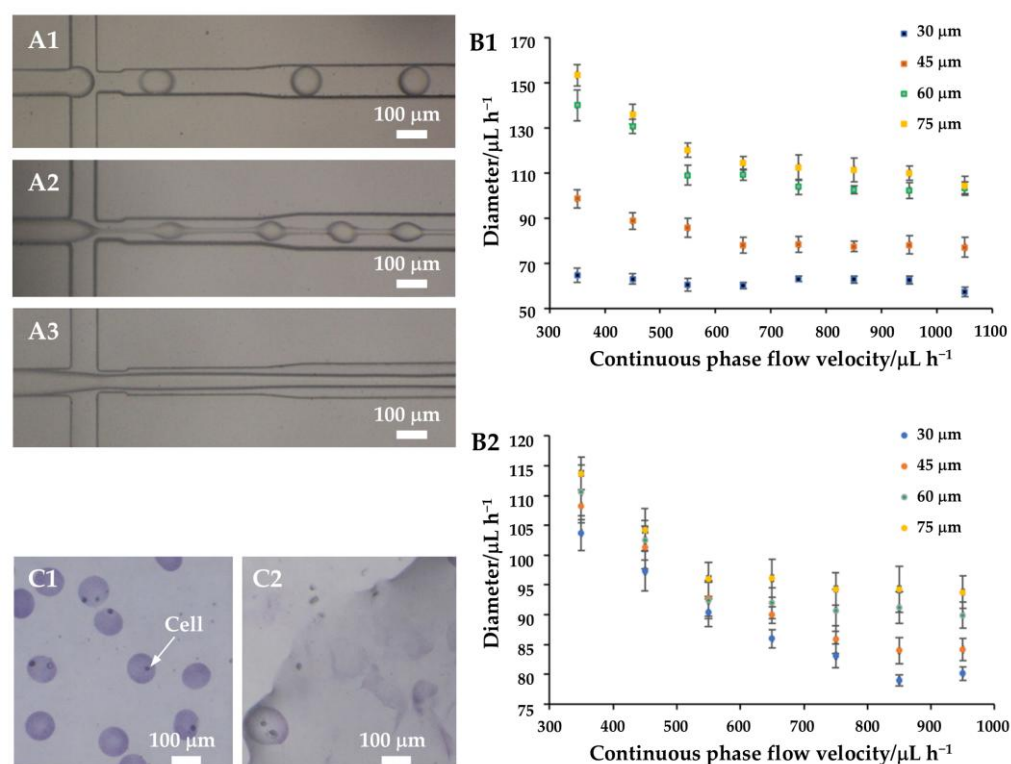


Figure 5. Effect of HAc concentration on droplet and microsphere formation. (A) Variations in flow patterns: (A1) droplet morphology, (A2) beads-on-a-string phenomenon, and (A3) filament formation. (B) Diameter of gel microspheres produced on chips of different widths at a fixed dispersed phase flow rate of 30 μL/h with varying continuous phase flow rates: (B1) 0.2% (*v/v*) and (B2) 0.1% (*v/v*) HAc concentrations. (C) Effect of HAc concentration on cell-laden gel microspheres: (C1) 0.2% (*v/v*) and (C2) 0.1% (*v/v*) HAc concentrations.

When the acetic acid concentration in the continuous phase was adjusted from 0.2% (*v/v*) (Figure 5B1) to 0.1% (*v/v*) (Figure 5B2), the diameter of stably generated gel microspheres increased significantly with every 15 μm increment in channel width, with the size being primarily constrained by the channel width. At a channel width of 30 μm, the droplet size was virtually unaffected by variations in continuous phase flow rate, producing gel microspheres with a diameter of 65 ± 5 μm. At a channel width of 45 μm, the gel microsphere diameter decreased with increasing continuous phase flow rate, varying within the range of 80–100 μm. Microfluidic chips with channel widths of 60 μm and 75 μm produced gel microspheres exceeding 130 μm in diameter under continuous phase flow rates below 450 μL/h, which are less suitable for cell culture. When the continuous phase flow rate exceeded 550 μL/h, the gel microsphere diameters ranged between 105

and 120 μm . The diameter of cell-laden gel microspheres affects the diffusion of substances from the environment into the microspheres; larger particle sizes require longer diffusion times. Therefore, under conditions that permit cell encapsulation, selecting smaller gel microsphere diameters enables more efficient delivery of oxygen and nutrients to the encapsulated cells, while metabolic byproducts produced by the cells can also be more easily expelled from the gel matrix, facilitating long-term culture. However, microsphere size also impacts cell encapsulation efficiency as well as the array immobilization and analysis of microspheres, necessitating comprehensive consideration in parameter selection.

Sodium alginate is a linear polysaccharide that can be crosslinked by divalent cations to form hydrogels. The concentrations of sodium alginate and Ca-EDTA solution significantly influence the mechanical strength of the gel microspheres. Typically, a 1:1 ratio of sodium alginate to Ca-EDTA solution yields the maximum product quantity. When the ratio deviates from 1:1, excess alginate anions or calcium ions remain, reducing both the yield and quality of the product. At low HAc concentrations in the continuous phase, the chelated calcium ions in the Ca-EDTA solution are not fully released. This incomplete gelation results in a loose alginate chain network structure prone to swelling and deformation, leading to reduced mechanical strength and increased diameter of the gel microspheres. Regarding the effect of channel width on microsphere size, at an HAc concentration of 0.1% (*v/v*), the diameters of gel microspheres fabricated using different channel widths varied significantly. In contrast, at an HAc concentration of 0.2% (*v/v*), all chelated calcium ions in the Ca-EDTA solution were released and bound to alginate anions, resulting in complete gelation. The resulting microspheres maintained excellent morphology, with diameter differences of less than 20 μm among the four channel sizes tested. Five replicate experiments were conducted at HAc concentrations of 0.1% (*v/v*) and 0.2% (*v/v*) under otherwise identical conditions (channel width: 75 μm ; dispersed phase flow rate: 30 $\mu\text{L/h}$; continuous phase flow rate: 600 $\mu\text{L/h}$; K562 cell density: 2×10^6 cells/mL). Statistical analysis was performed on the mean values and standard errors of gel microsphere size uniformity, single-cell encapsulation efficiency, and cell viability. At an HAc concentration of 0.2% (*v/v*), the generated gel microspheres exhibited superior size uniformity and single-cell encapsulation efficiency, while cell viability remained relatively unaffected by HAc concentration (Table 1). This is attributed to the fact that lower HAc concentrations in the continuous phase cause excessive swelling of the prepared gel microspheres, resulting in reduced mechanical strength. These microspheres are prone to adhesion and deformation, leading to size heterogeneity (Figure 5C). At an HAc concentration of 0.1% (*v/v*), a small number of cells were observed scattered outside the gel microspheres in the culture dish. Excessive swelling caused partial leakage of cells from the microspheres, resulting in decreased encapsulation efficiency (Figure 5C2). When serving as three-dimensional cell culture units, gel microspheres must maintain stable mechanical and chemical properties in aqueous media (such as buffers or cell culture media) along with good biocompatibility. Gel microspheres prepared with 0.2% (*v/v*) HAc exhibited higher crosslinking density, minimal water absorption and swelling, better structural integrity, and higher single-cell encapsulation efficiency and maintained high cell viability (Figure 5C1). Therefore, the following optimized conditions were selected: continuous phase HAc concentration of 0.2% (*v/v*), channel width of 75 μm , dispersed phase flow rate of 30 $\mu\text{L/h}$, and continuous phase flow rate of 600 $\mu\text{L/h}$. Under these conditions, the production rate of cell-laden microspheres reached 7 microspheres per second, with a microsphere diameter of 95 μm and a coefficient of variation (CV) of 3.85%.

Table 1. Effect of HAc concentration on the preparation of cell-laden gel microspheres.

HAc Concentration	Single-Cell Encapsulation Efficiency		Cell Viability		Size Uniformity	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
0.1% (v/v)	19.52%	0.011	96.04%	0.027	5.99%	0.016
0.2% (v/v)	22.31%	0.010	95.10%	0.015	3.85%	0.009

3.1.6. Effect of Cell Density on Cell Encapsulation

Mixed solutions were prepared with HFE-7100 containing 5% (v/v) PFO and 0.2% (v/v) HAc as the continuous phase. Four groups of prepolymer solutions with different K562 cell densities were employed as the dispersed phase for the fabrication of cell-laden gel microspheres using a 75 μm microchannel. The flow rates of the continuous and dispersed phases were fixed at 600 $\mu\text{L/h}$ and 30 $\mu\text{L/h}$, respectively. Cell encapsulation in the gel is a stochastic process directly correlated with cell density. When utilizing high-concentration cell suspensions (10^7 cells/mL) for gel encapsulation, cell aggregation readily occurs within the channel, often resulting in microsphere-induced channel clogging, failed encapsulation, or poor encapsulation outcomes. Single-cell encapsulation efficiency is primarily governed by microsphere size and cell suspension density. Once the microsphere dimensions are established, cell loading can be modulated by diluting the cell suspension.

Four groups (Group I, II, III, and IV) of dispersed phases containing K562 cells at densities of 2.3×10^6 cells/mL, 3.5×10^6 cells/mL, 4.8×10^6 cells/mL, and 6.5×10^6 cells/mL were prepared, respectively. Cell encapsulation experiments were conducted three times for each group, and the results are shown in Figure 6. In Group I, $73.6\% \pm 2.8\%$ of the gel microspheres were empty, $22.1\% \pm 2\%$ contained single cells, $4.3\% \pm 1.2\%$ contained two cells, and no microspheres containing three or more cells were observed. In Group II, $54.2\% \pm 2.1\%$ of the microspheres were empty, $28.6\% \pm 1.3\%$ contained single cells, $14.7\% \pm 1\%$ contained two cells, and $2.5\% \pm 0.9\%$ contained three or more cells. The proportion of empty microspheres decreased significantly, while the single-cell encapsulation efficiency increased markedly. In Group III, $47.7\% \pm 2.1\%$ of the gel microspheres were empty, $33.8\% \pm 1.5\%$ contained single cells, $13.4\% \pm 1.8\%$ contained two cells, and $5.1\% \pm 0.9\%$ contained three or more cells, demonstrating favorable single-cell encapsulation performance. Due to the significantly increased cell density, the proportion of microspheres containing three or more cells increased noticeably, with some microspheres containing a larger number of cells appearing. The proportion of empty microspheres decreased further, and the percentage of single-cell-laden microspheres increased further, though with a smaller increment, as the encapsulation efficiency was approaching the theoretical limit of approximately 37%. Further improvement in single-cell encapsulation efficiency would require more extensive density gradient analysis, which would be of limited significance. For Group IV, due to the excessive cell concentration, cell aggregation, adhesion, and channel clogging occurred, preventing stable droplet generation.

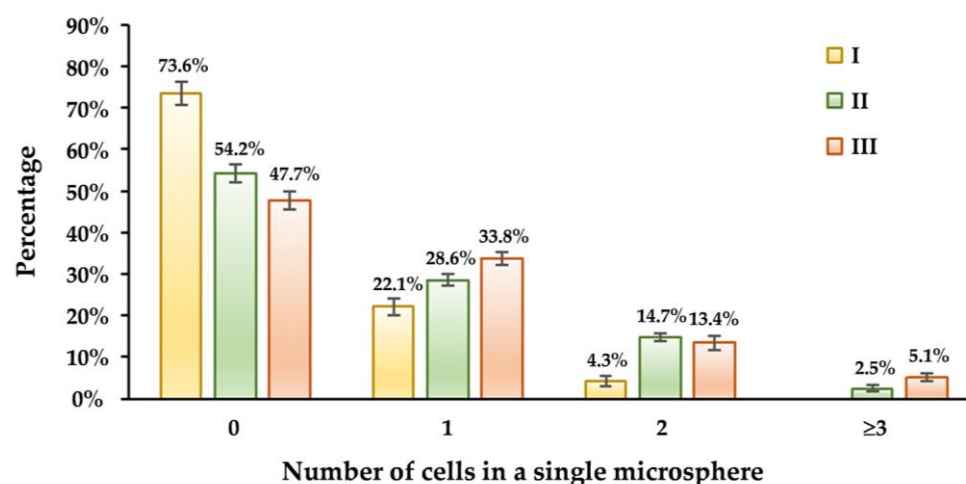


Figure 6. Distribution of cell encapsulation efficiency in gel microspheres fabricated using various cell densities under consistent preparation parameters.

3.2. Microsphere Capture and Cell Culture

After formation of the cell-laden microspheres, a cell culture medium was prepared by supplementing DMEM with 10% (*v/v*) fetal bovine serum (FBS) and 1% (*v/v*) penicillin-streptomycin. This medium was introduced into the chip as the wash phase to neutralize the pH of the microsphere preparation fluid and provide necessary nutrients for the cells. To facilitate visualization of the transparent gel microspheres, an appropriate amount of Nile blue stain was added to the culture medium. Once the trapping structure was filled with gel microspheres, the continuous and dispersed phase inlets were stopped and sealed. The wash phase flow rate was then reduced, allowing continuous perfusion over the fixed microsphere array. Subsequently, the chip surface was disinfected with ethanol and transferred to a CO₂ incubator. The culture medium inlet was connected to a syringe pump via polytetrafluoroethylene (PTFE) tubing, and medium was infused at a flow rate of 10 µL/h for *in situ* cell culture.

3.2.1. Effect of Wash Phase Channel Configuration

The generated gel microspheres flowed along the channel into the capturing module to form a microsphere array. The wash phase solution neutralized the pH of the preparation solution, preventing cells from prolonged exposure to acidic environments. Meanwhile, continuous perfusion of culture medium ensured adequate nutrient supply for the cells. In the preliminary design, a unilateral wash phase channel was connected downstream of the serpentine channel in the preparation module (Figure 7A), intersecting the serpentine solidification channel at a 35° angle to minimize backward thrust on the solution and gel microspheres within the serpentine solidification channel after the washing solution merged.

Upon entering the main channel from the unilateral side, the wash phase formed a stratified flow with the continuous phase due to the immiscibility of the aqueous and oil phases. Owing to the hydrophilic nature of the gel microspheres, those originally flowing at the center of the serpentine channel were squeezed toward the culture medium side. The compression of the aqueous phase by the oil phase, combined with the narrow channel dimensions, caused the microspheres to readily contact and adhere to the channel walls. Due to viscous effects, the velocity profile in the channel was parabolic—the maximum value was observed at the center and it decreased toward the walls. The channel walls exerted a hindering effect on the adhered gel microspheres, leading to uncontrollable accumulation (Figure 7B). Therefore, the connection configuration of the wash phase

channel must fully account for the forces acting on and spatial distribution of the microspheres. To mitigate the wall adhesion issue, the channel downstream of the junction was appropriately widened (Figure 7C). However, as the fluid entered the expanded channel, the flow velocity decreased instantaneously, causing the gel microspheres to merge into and accumulate within the aqueous plugs/slugs formed by the wash phase. The oil phase failed to propel these clustered microspheres, resulting in complete channel blockage (Figure 7D).

The use of a unilateral wash phase channel inevitably resulted in microsphere accumulation and clogging in the downstream channel. The improved design employed a symmetric washing configuration, aiming to maintain microspheres in the center of the channel via sheath flow and achieve liquid exchange. The downstream channel at the junction was appropriately widened to prevent gel microspheres from contacting the channel walls, while avoiding excessive width that would induce microsphere accumulation (Figure 7E). Upon entering the downstream channel, the gel microspheres remained positioned at the center, with the wash phase fluids on both sides exerting symmetrical hydrodynamic forces to maintain stable central flow (Figure 7F). The gel microspheres merged into the wash phase to form monodispersed aqueous droplets containing multiple microspheres, which flowed in a dispersed and orderly manner along the centerline.

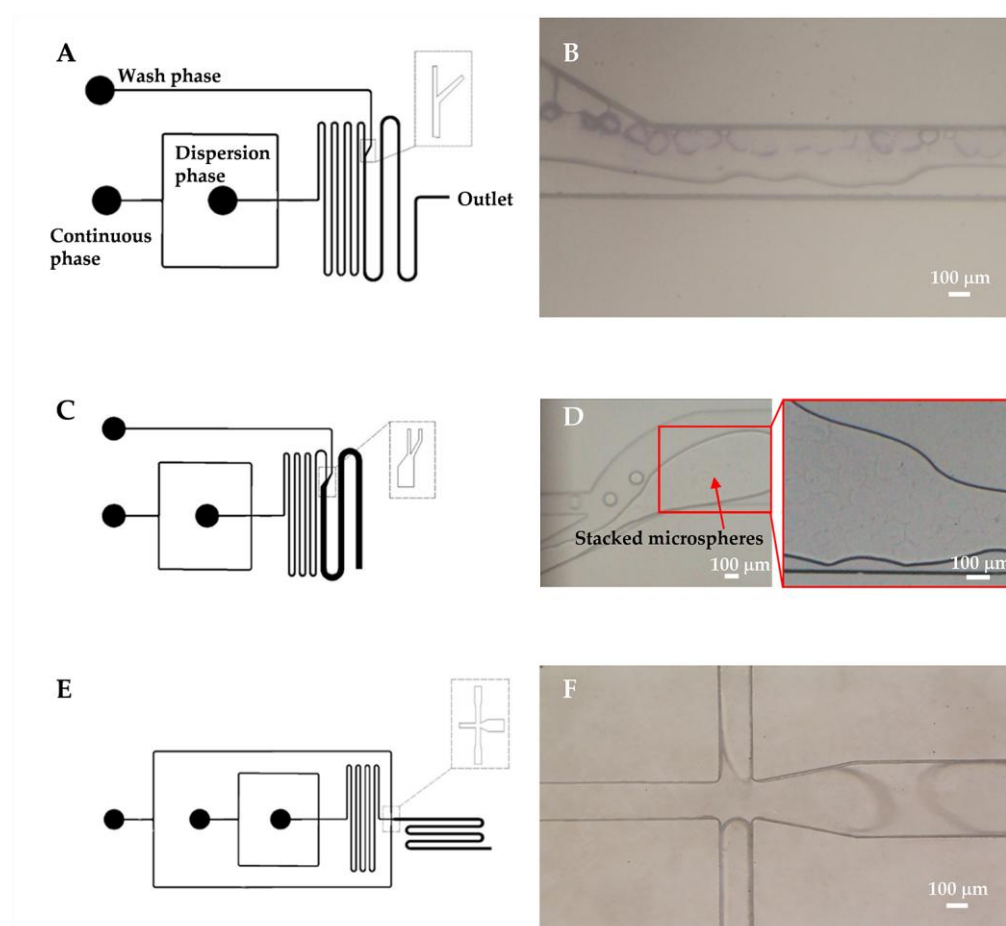


Figure 7. Effect of wash phase channel configuration on microsphere flow. (A) Schematic of the microfluidic chip with unilateral wash phase channel and (B) channel clogging during gel microsphere preparation. (C) Schematic of the microfluidic chip with widened unilateral wash phase channel and (D) channel clogging during gel microsphere preparation. (E) Schematic of the microfluidic chip with symmetric wash phase channels and (F) orderly droplet flow during gel microsphere preparation.

3.2.2. Cell Positioning and Culture on Chip

The cell capturing and culture module traps cell-laden hydrogel microspheres in specific regions of the chip to form arrays, while enabling microsphere-based compartmentalized cell culture. The hydrodynamic trapping structure (Figure 8A) consists of capture chambers positioned along the main channel. When a capture chamber is empty, the fluidic resistance of the main channel (R_1) exceeds that of the capture chamber (R_2), resulting in a higher flow rate through the capture chamber ($Q_2 > Q_1$). This causes microspheres to preferentially flow into and become trapped within the capture chamber. Once a microsphere blocks the chamber outlet, the fluidic resistance R_2 increases sharply, directing subsequent microspheres to continue along the main channel until captured at the next trapping site. The initially designed capture module comprises an array of 11×13 chambers positioned downstream of a serpentine main channel, with the array configuration adjustable based on microsphere quantity (Figure 8B). The prepared hydrogel microspheres exhibit a diameter of approximately $95 \mu\text{m}$ and possess certain mechanical elasticity. To ensure successful trapping while preventing channel clogging, individual chambers were designed with a width of $110 \mu\text{m}$ and length of $100 \mu\text{m}$, featuring an inlet width of $85 \mu\text{m}$ and a $15 \mu\text{m}$ -wide opening at the chamber bottom. The hydrogel microspheres are generated at an extremely rapid rate, moving through the channel in succession at intervals of approximately $300 \mu\text{m}$ per microsphere. To prevent channel blockage caused by the slow movement of these microspheres, the serpentine main channel width was set to $120 \mu\text{m}$.

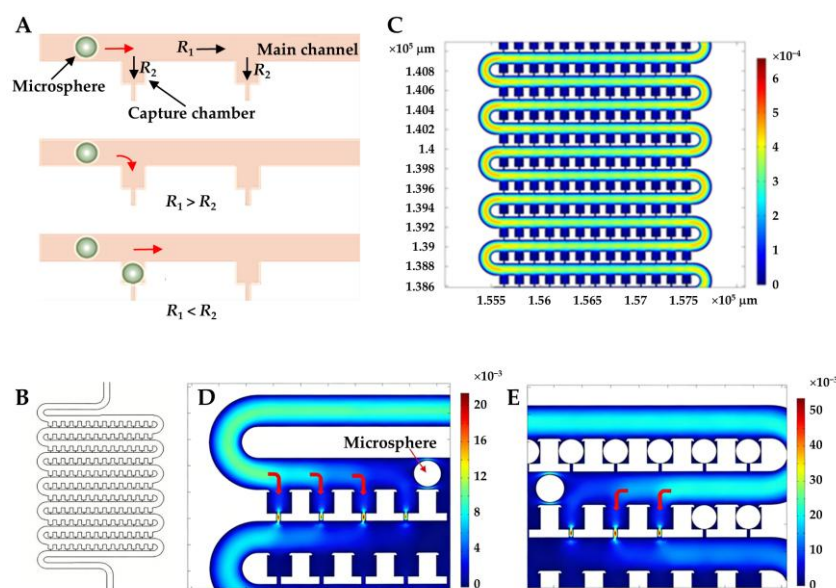


Figure 8. Hydrogel microsphere trapping and cell culture structure. (A) Working principle of hydrodynamic trapping of cell-laden microspheres. (B) Array configuration of capture chambers. (C) Computational simulation results of the trapping structure without considering microsphere dimensions. (D) Computational simulation results accounting for microsphere dimensions. (E) Effect on fluid flow following microsphere capture.

The trapping efficiency of hydrogel microspheres is governed by the flow resistance distribution within the channels. To elucidate this effect, computational simulations were conducted using COMSOL Multiphysics 5.6 (Burlington, MA, USA). The continuous phase flowed at $600 \mu\text{L/h}$ and the dispersed phase at $30 \mu\text{L/h}$, with channel cross-sectional dimensions of $120 \mu\text{m}$ (length) $\times$ $50 \mu\text{m}$ (width), yielding an inlet velocity of $1.58 \times 10^{-2} \text{ m/s}$. Initially, the microspheres were assumed to be sufficiently small so that they did not perturb the overall flow resistance distribution, and the fluid was therefore modeled as a homogeneous single-phase fluid. Under these conditions, the hydraulic resistance of the

serpentine channel was substantially lower than that of the capture chambers; consequently, microspheres preferentially followed the path of least resistance, with the majority exiting the chip via the serpentine channel (Figure 8C). However, during actual trapping operations, the microchannels contain numerous microspheres simultaneously, necessitating consideration of their impact on flow resistance. Due to the narrow width of the main channel, when a leading microsphere occupies the serpentine channel, it effectively functions as a plug for subsequent microspheres, significantly increasing the hydraulic resistance of the main channel. As the capture chambers now present relatively lower resistance with higher flow rates compared to the obstructed main channel, microspheres are diverted into vacant capture chambers (Figure 8D). Once a capture chamber is occupied by a microsphere, it maintains high flow resistance, causing subsequent microspheres to travel along the alternative path of lower resistance through the main channel (Figure 8E). Upon complete filling of all capture chambers, the flow velocity in the serpentine main channel increases markedly, enabling excess microspheres to be rapidly flushed out of the chip. Concurrently, the diminished flow velocity within the filled capture chambers helps reduce shear force-induced effects on the encapsulated cells.

3.2.3. Connection of Microsphere Preparation and Capture Modules

The cell-laden hydrogel microsphere capturing and cell culture module must be integrated with the microsphere preparation module to establish a complete fluidic circuit. Experimental results demonstrated that the incorporation of microfluidic structures at the downstream end of the serpentine channel did not significantly perturb the positive pressure-driven microsphere generation operated by the syringe pump. Consequently, the two modules can be directly interfaced via microchannels, with the capture module positioned immediately downstream of the microsphere preparation module (Figure 9A).

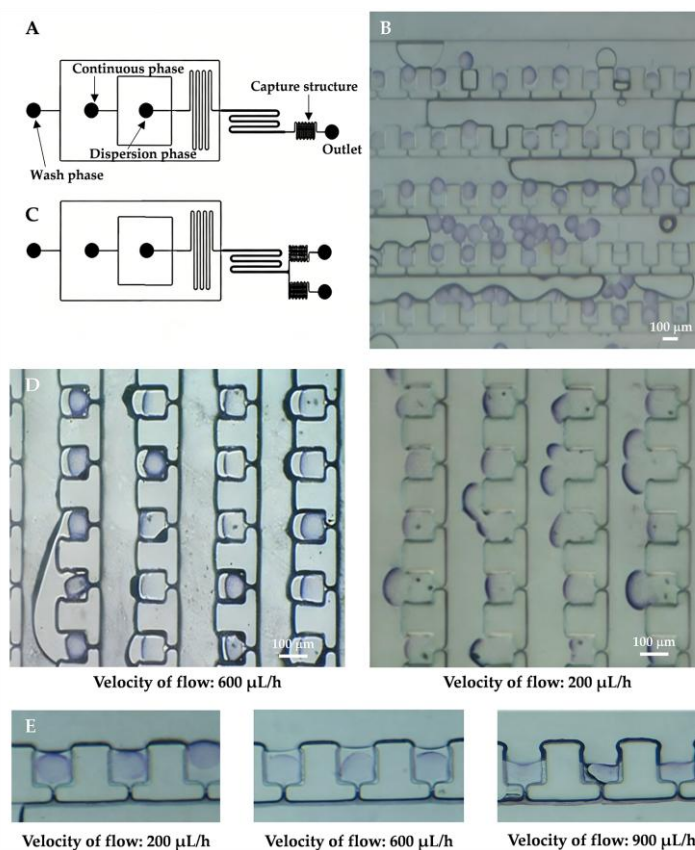


Figure 9. Microsphere capture and cell culture. (A) Microchip with single-trap structure. (B) Accumulation and clogging of microspheres in the single-trap structure. (C) Microchip with symmetric

trap structure. (D) Capture performance of gel microspheres at different washing-phase flow rates. (E) Status of immobilized gel microspheres under various flow rates.

When the microsphere suspension transitions from the wider washing channel into the narrower capture structure, the serpentine channel width at this junction is merely 120 μm , leading to increased fluidic resistance. Owing to the extremely rapid microsphere generation rate, substantial microsphere accumulation occurs in the main channel of the capture structure within a short timeframe, eventually resulting in channel blockage over time (Figure 9B). To mitigate this issue, two symmetric microsphere capture modules were constructed downstream (Figure 9C), effectively halving both the flow rate and microsphere throughput for each individual capture and culture module during the same interval. This approach reduces clogging risks without necessitating a decrease in generation flow rates, thereby preserving the integrity of the generation process and microsphere yield. Simultaneously, the incorporation of symmetric modules doubles the total number of capture units to 286.

3.2.4. Continuous Microsphere Preparation and Capture

The wash-phase flow rate is the primary factor governing the movement of liquid and microspheres within the downstream capture module, significantly affecting capture time, capture stability, and the occurrence of channel clogging. Microsphere capture was evaluated at wash-phase flow rates of 30 $\mu\text{L/h}$, 200 $\mu\text{L/h}$, and 600 $\mu\text{L/h}$. At 600 $\mu\text{L/h}$, microspheres were immobilized in nearly every capture unit without instances of multiple microspheres being trapped in a single unit (Figure 9D). At 200 $\mu\text{L/h}$ and 30 $\mu\text{L/h}$, the velocities of both the liquid and microspheres decreased markedly. Although microsphere capture occurred in each chamber, the proportion of units trapping multiple microspheres increased substantially (approximately 50%).

During perfusion of cell culture medium, maintaining a consistently high flow rate may cause immobilized microspheres to escape from the capture units due to excessive deformation; therefore, the stability of gel microspheres under different flow rates was evaluated. The culture medium flow rate was increased from 10 $\mu\text{L/h}$ to 100 $\mu\text{L/h}$, followed by gradual increments of 100 $\mu\text{L/h}$ up to 1000 $\mu\text{L/h}$, with a duration of 5 min for each step. At flow rates ranging from 10 $\mu\text{L/h}$ to 400 $\mu\text{L/h}$, the gel microspheres exhibited neither escape nor significant deformation (Figure 9E). When the flow rate was increased to 600 $\mu\text{L/h}$, partial compression and deformation of some gel microspheres was observed. Upon reaching 900 $\mu\text{L/h}$, the gel microspheres underwent severe compressive deformation and overflowed from the rear openings. As the flow rate progressively increased, the deformation of gel microspheres became more pronounced, indicating that microspheres cannot be subjected to high-flow environments for extended periods. During microsphere preparation, the total flow rate was 1230 $\mu\text{L/h}$, and the capture structure was completely filled within 2 min after droplet generation. Consequently, the dispersed phase and continuous phase inlets can be sequentially stopped, followed by reduction in the wash-phase (culture medium) flow rate. Strict control of the preparation timing is essential to prevent cell escape resulting from severe microsphere deformation.

3.2.5. On-Chip Cell Culture

The microfluidic chip, prepared continuous phase solution, dispersed phase prepolymer solution, and syringes were placed in a laminar flow hood and sterilized under UV irradiation for 20 min. System assembly was completed in the laminar flow hood; the sealed microchip effectively isolated bacteria and contaminants. Fluorinated oil exhibits extremely high oxygen solubility, which facilitates O_2 transport in PDMS channels during the preparation phase. Additionally, PDMS material offers excellent optical transparency

and high permeability to both O₂ and CO₂. During cell culture, the CO₂ incubator was set to 37 °C with 5% CO₂ concentration to meet experimental requirements. Cell culture medium containing 10% fetal bovine serum and 1% penicillin-streptomycin was used as the third-phase solution for the microfluidic chip. Cell culture experiments were conducted under identical parameter conditions, followed by live/dead cell staining after 24 h of culture. Among 286 gel microsphere trapping units, the single-cell encapsulation rate was $30.87 \pm 1.63\%$, with a total of 174 ± 2 captured cells. After 24 h of culture, the cell viability was $95.21 \pm 2.69\%$, achieving high single-cell encapsulation efficiency and cell survival rates.

4. Conclusions

To address the limitations of low automation and integration in existing microfluidic chips for single-cell culture using gel microspheres as carriers, this study integrates functions including cell-laden gel microsphere generation, trapping, and culture onto a single chip, achieving single-cell gel encapsulation and cell culture analysis. Through particle size measurement and analysis of gel microspheres generated under different HAc concentrations, an optimal HAc solution concentration (0.2% (v/v)) was determined. Meanwhile, by analyzing flow patterns in microchannels and comparing gel microsphere generation under different channel widths and flow rate ratios, the optimal microchannel width was determined to be 75 µm. With continuous and dispersed phase flow rates of 600 µL/h and 30 µL/h respectively, and a cell density in the dispersed phase of approximately 4.8×10^6 cells/mL, droplets could stably generate and fully solidify into monodisperse gel microspheres (average diameter of 95 µm, CV = 3.85%) with favorable cell encapsulation efficiency. Experiments demonstrated that the integrated chip exhibited excellent microsphere generation and trapping capabilities, achieving a single-cell loading rate of 33.8% for immobilized gel microspheres. By controlling culture medium composition, flow rate, and environmental temperature during cell culture, K562 cell culture was successfully realized. At an environmental temperature of 37 °C, CO₂ concentration of 5%, and medium perfusion rate of 10 µL/h, cell viability exceeded 95% after 24 h. Cell culture experiments confirmed that the chip possesses good biocompatibility and is suitable for on-chip in situ single-cell culture of various cell types, providing a novel platform for single-cell culture and analytical detection. The new device simplifies single-cell culture workflows, enhances research efficiency, and demonstrates promising application prospects in fields including cellular heterogeneity research, cell therapy, and tissue engineering. Future work will focus on further improving single-cell encapsulation efficiency and integrating real-time biosensing modules for dynamic cellular analysis.

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