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One-step wettability patterning of PDMS microchannels for generation of monodisperse alginate microbeads by in Situ external gelation in double emulsion microdroplets

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ABSTRACT

Droplet-based microfluidic systems are promising tools for many biological applications from digitalized analysis to drug delivery and biomolecules synthesis. Among these, fabrication of microgels, particularly alginate microbeads, has substantial importance due to their capability for three-dimensional (3D) encapsulation of cells, drugs and other bioactive materials. However, biocompatible generation of monodisperse alginate microbeads is still challenging. In this study, a new droplet-based microfluidic strategy is developed for cell and chip friendly fabrication of monodispersed alginate microbeads. The polydimethylsiloxane (PDMS) microfluidic device is first treated using on-chip plasma-assisted deposition of polyvinyl alcohol, to selectively modify the hydrophobicity of microchannels. The proposed wettability patterning approach is permanent, simple, reliable, and time-effective, which makes the resulting microfluidic device highly stable and controllable for generation of double emulsions (DEs). The device is then used for fabrication of monodisperse alginate microbeads using external gelation in DE microdroplets. The reported microbead fabrication method offers both biocompatibility as a result of external gelation and rapid recovery of beads from processing solutions, as well as chip-compatibility due to clog-free gelation. This simple and robust DE strategy provides new opportunities not only for 3D micro-encapsulation but also for other droplet-based microfluidic applications in diagnostic testing and drug delivery.

1. Introduction

Engineering hydrogels has emerged as a promising approach in biomedical applications for three dimensional (3D) encapsulation of cells [1,2] and bioactive materials [3,4]. Alginate is one of the widely used hydrogels for drug delivery, tissue engineering and wound healing [5,6]. Biocompatibility of the solutions, hydrogel and degraded forms as well as simple and immediate ionotropic gelation, and finally tunability of mechanical and physical properties make alginate a favorable bio-scaffold [7,8]. Alginate microgels can provide 3D discrete microenvironments which may be manipulated or monitored independently. Such structures could be used for example in cell spheroid formation [9,10] and co-culture of different cell types [11,12]. The microgels enhance cell-cell and cell-matrix interactions which are crucial for recapitulating in vivo cellular behaviors such as differentiation of stem cells [13,14] and resistance of tumor cells against mechanical stresses, immune attack and chemotherapies [15,16]. Alginate microgels also offer immunosuppression-free transplantation due to their capability to provide cell immunoisolation [17,18]. For these applications, alginate microgels need to provide some key specifications. First, the size of microgels should be small-enough to establish a homogeneous structure upon crosslinking and allow effective transportation of nutrient, oxygen and wastes. Second, their size distribution is necessary to be monodispersed to ensure reliability and repeatability of the upstream characterization due to similar number of encapsulated cells and cell-cell interaction. Finally, a uniform spherical shape of the microgels is preferred for providing homogeneous internal microenvironment and improved capability of implantation [19,20].

The process of fabricating microgels involves dispersion of alginate solution followed by gelation for establishment of solid networks [21,22]. Against traditional methods, microfluidic platforms are capable of generating monodisperse small spherical droplets from alginate

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solution with high accuracy, reproducibility and throughput [23,24]. In these platforms, the aqueous alginate stream is usually broken into discrete volumes by an immiscible oil phase. Consequently, the generated alginate droplets need to be gelled using divalent cations, mainly Ca^{2+}. Because calcium salts cannot be dissolved in oil, external delivery of Ca^{2+} ions to alginate droplets dispersed in continuous oil phase is challenging. Usually droplets after generation are introduced into a gelation bath, containing Ca^{2+} ions. However, the alginate micro-droplets either cannot break the covering oil/water interface to reach the gelation bath, or the passage of the droplet through the interface cause the gradual crosslinking of alginate droplet and creation of tailed-shape particles [10,25]. This drawback could be resolved by application of internal gelation approaches where either water-insoluble calcium carbonate ($CaCO_3$) nanoparticles [26,27] or calcium-chelated ethylenediaminetetraacetic acid (EDTA) [11,28] is used in alginate solution. Upon decreasing the pH, usually by incorporating acetic acid dissolved in oil phase, Ca^{2+} ions are released and form a gelled network. The challenge of gelation could be resolved in this method but with the costs of toxicity by acidic condition and possibility of clogging the channels by aggregation of gelling droplets. Therefore, a proper microfluidic-compatible gelation method, preferentially based on external crosslinking, is still an important yet unmet demand.

Moreover, toxicity has shown to be induced even after gelation, as harvesting the microgels usually need extensive time and labor consuming washing steps which prolongs the oil and surfactant exposure and decreases the viability of the cells [29,30]. Alternatively, double emulsion (DE) water-in-oil-in-water (W/O/W) droplet generation approach has been used to minimize the oil and surfactant exposure [31,32]. Nevertheless, to generate both W/O and O/W droplets in a single microdevice, either coaxial capillary platforms [33,34] are required or the microfluidic channels need surface modification to manipulate their wettability. Various methods have been developed for permanent modification of PDMS wettability, including deposition of plasma polymerized coatings [35], sol–gel glass coating [36], photolithography-based photoreactive sol-gel coating [37], and layer-by-layer deposition of positively and negatively charged species [38]. However, complex fabrication process and reagent preparation limited the application of these approaches. Alternatively, oxidization using plasma and UV exposures have been used to make PDMS surfaces hydrophilic [39]. Although simple, the effect of these surface treatments is transient and barely compatible for selective wettability patterning to generate DE in a single microfluidic chip. To make the hydrophilicity permanent, selective coating of polyvinyl alcohol (PVA) on PDMS surfaces following to plasma treatment may be used [40,41]. Although this method offers a simple, quick and cost effective wettability patterning for DE droplet generation, selective coating of PVA solution in desired microchannels is challenging, particularly after plasma treatment.

Here we introduce a one-step, on-chip wettability patterning using a modified PVA deposition approach for generation of DE droplets within a microfluidic chip. Compared to previous methods, selective coating of microchannels is highly stable, controllable and rapid. We then exploit the DE droplets for highly biocompatible fabrication of alginate microbeads, using external gelation with minimum exposure of oil and surfactant. DE strategy further offers a clog-free approach for high-throughput microfluidic-based generation of monodisperse microgels, essential for further high-throughput cell, tissue and drug analysis.

2. Materials and methods

2.1. Materials

SU-8 2050 was purchased from MicroChem Corporation. SYLGARD® 184 silicone elastomer kit (including PDMS base and curing agents) was obtained from Dow Corning. Sodium alginate, calcium chloride, M4810 mineral oil, Span® 80, Pluronic® F-127 and 87–90% hydrolyzed PVA were purchased from Sigma-Aldrich. Dulbecco's phosphate buffer saline (DPBS, Gibco), Hank's balanced salt solution without calcium and magnesium (HBSS, Gibco), minimum essential medium (MEM, Gibco), fetal bovine serum (FBS, Gibco), and penicillin/streptomycin (Gibco) were used for cell culture experiments. Trypan Blue (Sigma-Aldrich) was used to examine viability of the cells.

2.2. Microfluidic device fabrication and wettability patterning

The design of the microfluidic device used in this study is shown in Fig. S1. The device was fabricated using the conventional photo- and soft-lithography methods [42]. Briefly, a master mold was prepared by patterning SU-8 on a silicon wafer using photolithography. The PDMS mixture (with 10:1 ratio of base:curing agents) was then poured on the mold, degassed and baked for 30 min at 80 °C. PDMS was then peeled off from the mold and punched for opening desired inlets/outlet using a 1 mm biopsy punch (KAI Industries). After cleaning with scotch tape, the PDMS was irreversibly bonded to glass slide by treatment of bonding surfaces using a laboratory corona treater (BD-20AC, Electrotechnic Products) for 1 min at 40 kV. The bonded device was then baked for 1 h at 80 °C and kept in Petri-dish for more than 2 days to completely retrieve its hydrophobicity.

Wettability patterning was performed using a modified PVA deposition approach (Schematic shown in Fig. S2). PVA was dissolved in distilled water (1 wt%) by multiple steps of vortex mixing and heating at 80 °C. To selectively modify the wettability of the microchannels, the PVA solution was injected into those channels that should be maintained hydrophobic (W/O droplet generation section of the chip). The flow rate of PVA solution was set to 10 μL min^{-1} using a syringe pump (AL-1000, World Precision Instruments). Simultaneously, air was passed at high flow rate (300 μL min^{-1}) through another inlet using a metallic connector. By connecting the electrode tip of the corona treater device to the metallic connector, the desired microchannels were plasma-treated. Applying both PVA solution and plasma treatment simultaneously at the output channels (O/W droplet generation section) causes permanent PVA deposition. On the other hand, passing PVA solution through W/O droplet generation section prevents air-blowing plasma treatment at these channels. After 5 min, the electrode was disconnected and PVA injection was stopped while the air was kept passing to remove PVA from all channels. Finally, PVA residuals were removed by air blowing, the device was heated for 10 min at 100 °C, and channels were briefly washed by injection of distilled water.

2.3. Generation of double emulsion droplets and formation of alginate microbeads

Sodium alginate (2 wt% in HBSS) solution was used as inner aqueous (IA) phase. To disperse alginate and generate W/O droplets, Oil/$CaCl_2$ nanoemulsion was used as the middle oil phase. The nanoemulsion was prepared by mixing a 1:7 volumetric ratio of 0.7 g ml^{-1} $CaCl_2$ solution and light mineral oil containing 1.25% Span 80, using gentle shaking followed by 5 min sonication for two times. To generate O/W droplets, distilled water supplemented with 1.25 wt% Pluronic F127 was used as the outer aqueous (OA) phase. Span 80 and Pluronic F127 were used to stabilize IA and oil droplets. For visualizing purpose, food dyes were used in IA and OA solutions. The solutions were loaded in plastic syringes, connected to the microfluidic device using Tygon® tubing (with internal diameter of 0.5 mm, Cole-Parmer), and injected at controlled flow rates using syringe pumps. Unless otherwise stated, the flow rates of IA, Oil and OA phases was set to 1, 2, 7 μL min^{-1}, respectively. DE droplet generation was examined using an inverted Leica DM IL LED microscope. Droplets were collected in 1.5 ml Eppendorf tubes filled with culture medium supplemented with 2 wt% $CaCl_2$, and centrifuged (Labnet, C1301) at 6000 rpm for 30 s. The resulting microgels were then washed using Dulbecco's phosphate-buffered saline (DPBS) and kept in culture medium.

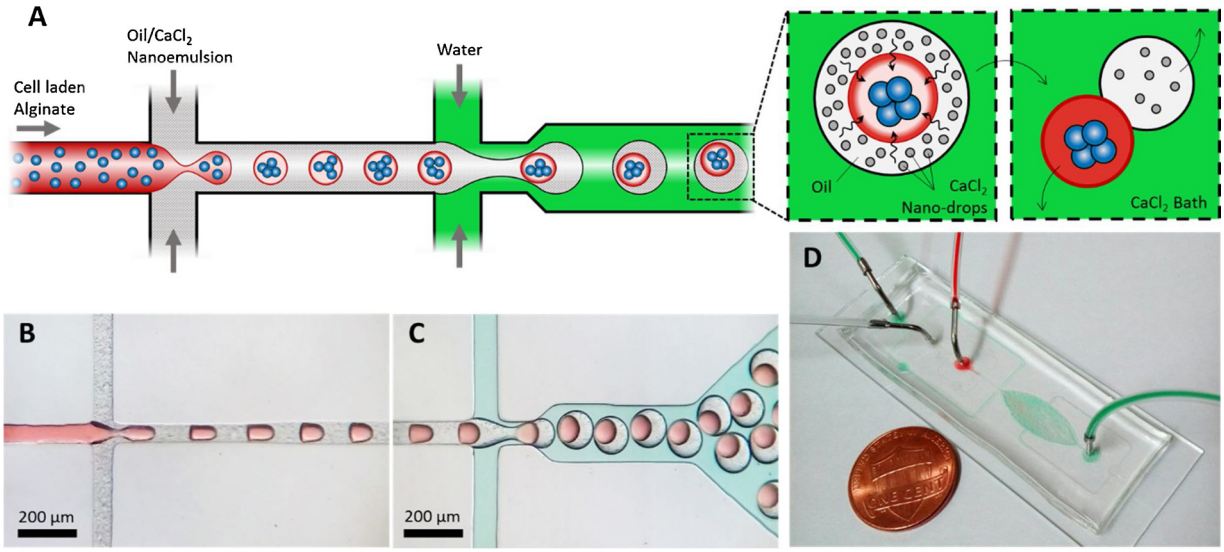

Fig. 1. Formation of alginate microbeads using two-step external gelation in double emulsion (DE) microdroplets. (A) Schematic illustration of cell-encapsulated microgel generation. In microfluidic device, cell-laden alginate droplets are formed using Oil/$CaCl_2$ nanoemulsion followed by their dispersion in the outer aqueous phase, generating double emulsion (DE) droplets. In DE system, $CaCl_2$ nano-drops infuse the alginate droplet and cause gelation of its surface. Subsequently, surface-gelled particle releases into $CaCl_2$ bath to accomplish gelation. (B) Generation of alginate and (C) DE droplets in the (D) real microfluidic device.

2.4. Cell culture and encapsulation

Human Embryonic Kidney 293 cells (HEK-293, Pasture Institute, Iran) were cultured in MEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained in T-75 flasks at 37 °C in a humidified 5% CO_2 atmosphere and subcultured at 80–90% confluency. For the encapsulation, cells were trypsinized and detached followed by resuspension at desired concentration (10×10^6–50×10^6 cell ml^{-1}) in alginate solution. Viability assessment of encapsulated cells performed using Trypan Blue reagent.

3. Results and Discussion

In this study, we propose a novel external gelation method for fabrication of monodispersed cell-encapsulated alginate microbeads (Fig. 1). As Agarwal et al. [43] suggested, $CaCl_2$ nano-drops emulsified in the oil phase can be used for external gelation of alginate microdroplets. First, we attempted to generate alginate microbeads using a similar single emulsion strategy, in which alginate droplets were dispersed in the oil phase and gelled by fusion of $CaCl_2$ nano-drops. Nevertheless, slow gelation process caused fusion of gelling alginate droplets and consequently raised the challenges of non-uniform size and shape of the microgels as well as clogging at the downstream outlets. A possible solution could be to increase the length of outlet channel for providing enough gelation time which in turn increases the required pressure, particularly due to high hydrodynamic resistance of viscous nanoemulsion, and causes leakage or unstable/backward flow in some channels. To tackle this challenge, we modified this approach to a more chip- and cell-friendly method using a two-step crosslinking in DE system. As depicted in Fig. 1A, alginate droplets were first generated using oil/$CaCl_2$ nanoemulsion (crosslinker oil). Lower concentration of $CaCl_2$ nano-drops compared to Agarwal et al. [43] nanoemulsions caused easy droplet generation, preventing premature gelation at the interface of alginate stream. Alginate droplets and their crosslinker oil were then dispersed in a second aqueous solution. The outer aqueous (OA) phase plays important roles in our system, including: (i) preventing fusion of gelling alginates that ensures monodispersity of resulting microgels and hinders clogging of the microfluidic device; (ii) decreasing hydrodynamic resistance of outer phase in microfluidic channels, and consequently dropping the injection pressure which stabilizes the functionality of the microfluidic chip; (iii) facilitating the release of microgel and minimizing oil and surfactant exposure that increases biocompatibility of the method for cell encapsulation. In DE droplets, the $CaCl_2$ nano-drops infuse the alginate droplet and cause partial gelation at its surface followed by complete gelation upon its release into $CaCl_2$ bath. Fig. 1B and C, respectively demonstrate W/O and O/W droplet generation within the employed microfluidic device (Fig. 1D).

3.1. Wettability patterning of microfluidic channels

The process of droplet generation in the microfluidic system described in previous section, depends on the wettability pattern of the microchannels. Because PDMS is inherently hydrophobic, it is an ideal target for W/O droplet formation, however the hydrophobicity prevents the generation of O/W droplets. Consequently, selective treatment is required to amend PDMS hydrophobicity in O/W droplet generation section of the microfluidic device. Recently, Trantidou et al. [41] introduced a protocol based on PVA deposition to make required channels hydrophilic, using manual injection of PVA solution in plasma-pretreated microfluidic device. We used a similar approach but with modification to make it much easier, faster and robust (Fig. 2). As demonstrated in Fig. 2A and schematically shown by Fig. S2, we performed the whole selective patterning of PDMS channels in one single step using simultaneous PVA injection and on-chip plasma treatment. Fig. S3 illustrates the effect of plasma and PVA treatment on wettability of PDMS surfaces. Plasma treatment increases the surface energy and generates alcoholic hydroxyl, silanol, and carboxylic acid radicals on the PDMS surface [44]. An estimation of surface energy (E) that can be obtained from contact angle (θ) measurements using the equation of state [45,46] indicates an increase from $E_0 \sim 20\ mJ\ m^{-2}$ (corresponding to the contact angle $\theta \sim 105^\circ$ of water on native PDMS) to $E_1 \sim 72\ mJ\ m^{-2}$ (corresponding to $\theta \sim 5^\circ$) due to plasma treatment. In this situation, PVA can be adsorbed irreversibly to PDMS as a result of covalent bonding of PVA molecules to plasma-generated radical species and create a stable PVA coating on the surface [44], with a decreased level of energy ($E_2 \sim 68\ mJ\ m^{-2}$ corresponding to $\theta \sim 20^\circ$). Consequently, both plasma and PVA treated PDMS surfaces permanently turn hydrophilic (Fig. S3A), however, the effect of sole plasma is transient. The diffusion of the uncured hydrophobic PDMS polymers from bulk to the surface, with a driving force to decrease the surface energy, can recover the surface to its original state over time (Fig. S3B). On the other hand,

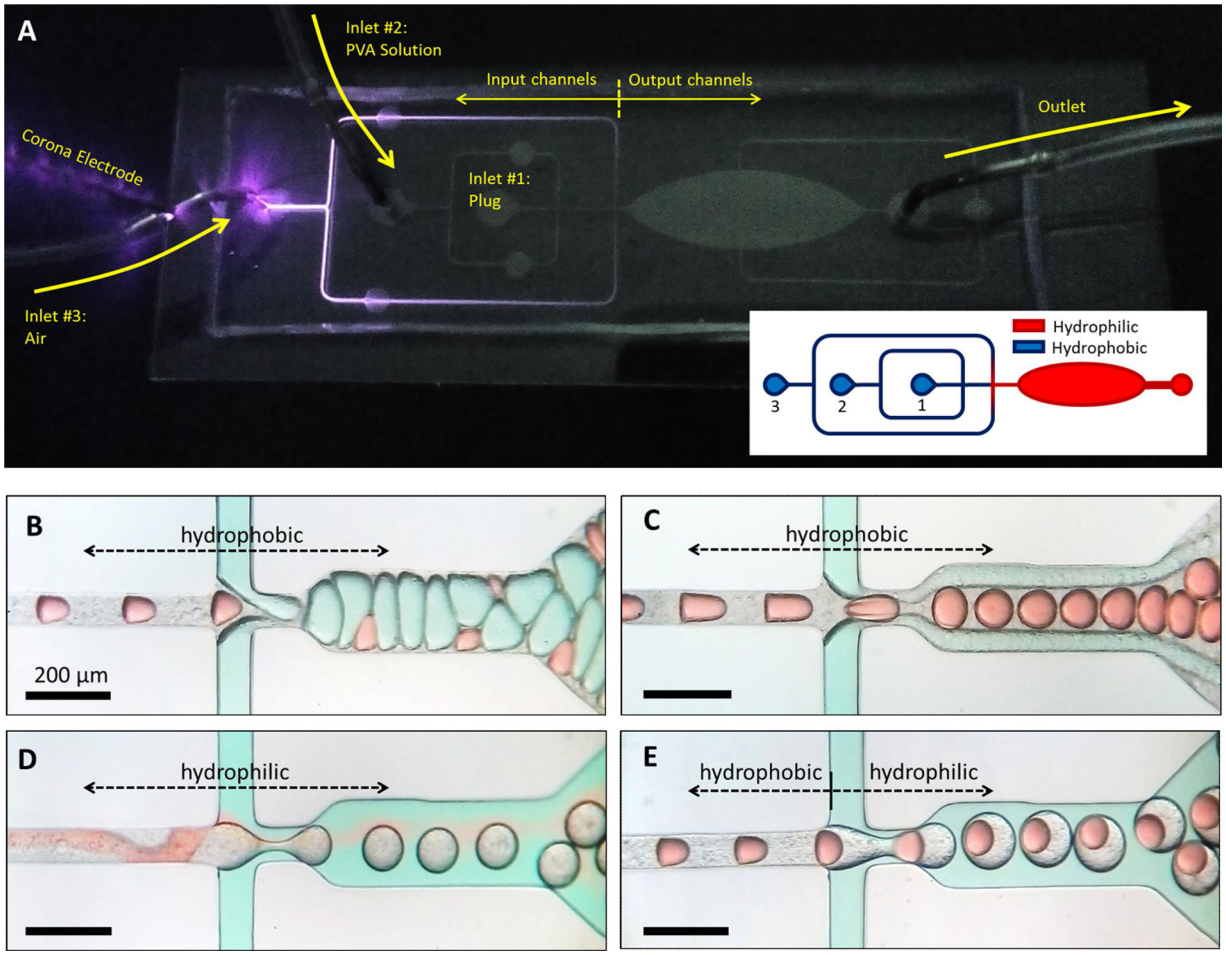

Fig. 2. Selective wettability patterning of polydimethylsiloxane (PDMS) microfluidic device for DE droplet generation. (A) One-step modification of desired channels' hydrophobicity using simultaneous PVA and plasma treatment. By connecting the electrode of a corona treater device to metallic connector passing air, plasma is injected into outer channel while PVA is injected into W/O droplet generation channels using middle (#2) inlet, both at controlled flow rates. As a result, only output channels are treated with both PVA and plasma that makes them permanently hydrophilic. Inset shows a permanent-wettability map after treatment. Evaluation of treatment after 10 days for (B) only-plasma treated device with normal flow rates (1, 2, 6 µL min^{-1} respectively for inner aqueous (IA, red), oil (white) and outer aqueous (OA, green) phases); (C) only-plasma treated device with increased IA and OA flow rates (2, 12 µL min^{-1}, respectively); (D) plasma and PVA treatment of the whole microfluidic network; and (E) selective plasma/PVA treated channels of the device using the procedure depicted in subfigure A (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

application of PVA without plasma treatment has insignificant effect on the hydrophobicity because PVA molecules cannot form covalent bonds to the surface and would be mostly removed by air blowing or washing (Fig. S3C).

The above described fact was used in our one-step wettability patterning protocol. To perform patterning, the microfluidic network was divided into input and output channels, in which the output channels were treated to be hydrophilic. The patterning was accomplished by injection of PVA and plasma into input channels. In our microfluidic device, PVA solution was injected through W/O droplet generation channels. Simultaneously, an innovative air-assisted plasma exposure exploited for treatment of OA channels by connecting the electrode of a corona treater device to the metallic connector injecting the air (Fig. 2A and S2).

Flow of air (plasma) and PVA solution hinders these fluids to enter each other channels, while the output channels are exposed to both plasma and PVA. Therefore, only output channels would be permanently hydrophilic. The PVA and air flow rates were adjusted to provide stable fluid flow as well as proper surface modification. Fortunately, foaming tendency of PVA solution facilitates the formation of stable fluid flow using a wide range of flow rates (Fig. S4). Within the stable range of flow rates, the microfluidic output channels turn uniformly hydrophilic. A uniform surface treatment can be obtained by multiple plasma and PVA treatment steps which provides a saturated PDMS surface with PVA molecules (Fig. S3D). The presented on-chip wettability patterning method can be considered as a sequence of many plasma and PVA treatment steps (Fig. S2) which guarantees a uniform wettability with the surface energy corresponding to a stable PVA coating throughout the output channels.

Various multiple emulsion droplets can be easily generated by designing the layout of input and output channels, as illustrated in Fig. S5, and tuning the flow rates. Furthermore, the wettability can be modified transiently by replacing PVA solution with water and adjusting flow rates accordingly. Because water cannot establish stable foam, the effect of plasma treatment can be identified from interference patterns (Fig. S6 and Video S1) generated as a result of roughness induced by plasma treatment [47,48].

To ensure selective wettability modification, droplet generation was evaluated after 10 days post-treatment (Fig. 2B–E). Despite facile generation of DE droplets immediately after treatment, the hydrophobicity was recovered after 10 days in plasma (and water) treated microfluidic devices. Therefore, the O/W droplets could not be formed (Fig. 2B) even by changing the input flow rates (Fig. 2C). On the other hand, manual injection of PVA solution after plasma treatment caused rapid

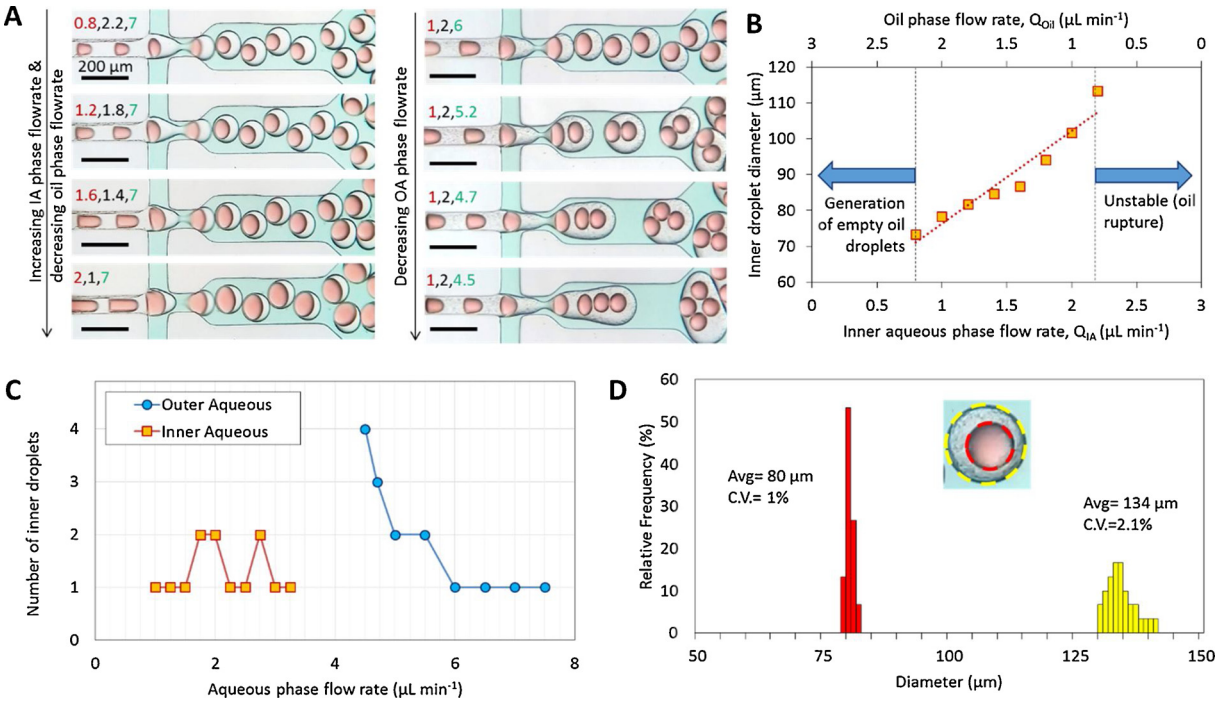

Fig. 3. Controlling morphology of the DE droplets. (A) Qualitative demonstration of control over the size of inner aqueous (IA) and oil droplets. Simultaneous increase in IA flow rate (Q_{IA}) and decrease in oil flow rate (Q_{Oil}) while keeping $Q_{IA}+ Q_{Oil}$ constant enlarges the core droplet independently. Alternatively, decreasing only outer aqueous flow rate (Q_{OA}) can increase the size of oil droplet and number of encapsulated IA cores. The numbers at the top-left of each figure show corresponding flow rates of IA, oil and OA phase in µL min^{-1}, respectively. (B) Quantitative representation of controlling the size of core droplet and (C) the number of encapsulated cores. (D) Size distribution of inner (red) and outer (yellow) droplets generated using 1, 2, 7 µL min^{-1} for Q_{IA}, Q_{Oil} and Q_{OA}, respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

filling of all channels, making the whole device hydrophilic in which the W/O droplets were not generated (Fig. 2D). Even by application of air pressure in W/O section while injecting the PVA solution (the method reported by Trantidou et al. [41]), the flow was uncontrollable due to high capillary force and compressibility of air, and consequently whole microfluidic network was turned hydrophilic in most of the devices (6/10). Finally, all devices (n = 10) prepared using our proposed protocol could reliably generate DE droplets (Fig. 2E, Video S2). As a result of high stability of PVA coating on PDMS surfaces (Fig. S3E), the devices could be stored for several weeks and applied for double emulsion generation for several hours. This simple, reliable and low-cost on-chip surface modification is a new technique that enables the controlled wettability patterning of complex microfluidic networks.

3.2. Controllable formation of double emulsion microdroplets

To demonstrate flexibility and controllability of the DE droplet generation, we investigated the effect of flow rates variation on morphology of DE droplets. The size of inner aqueous (IA) and outer oil droplets in the DE system could be manipulated while preserving their monodispersity (Fig. 3). In general, the size of generated droplets can be controlled by tuning the ratio of disperse and continuous phase flow rates [49]. In DE droplets, the size of inner and outer droplets could be adjusted independently to generate bigger or multiple cores, respectively (Fig. 3A).

To independently change the size of inner droplets, the ratio of IA phase flow rate (Q_{IA}) to oil phase flow rate (Q_{Oil}) is changed while keeping the ratio of $Q_{IA}+ Q_{Oil}$ to OA phase flow rate (Q_{OA}) constant. Fig. 3B illustrates the tunability of inner droplet diameter by modulating Q_{IA}/Q_{Oil} and setting Q_{OA} and $Q_{IA}+ Q_{Oil}$ constant at 7 and 3 µL min^{-1}, respectively. It is demonstrated that increasing Q_{IA}/Q_{Oil} enlarges the core droplets up to an extent that the DE droplets experience premature rupture as a result of IA phase contact with hydrophilic walls at the top and bottom of the channels. Furthermore, the size of inner droplets can be decreased to a minimum value after which empty oil droplets may be generated.

Alternatively, the size of oil droplets can be changed independently by controlling the $(Q_{IA}+ Q_{Oil})/Q_{OA}$ ratio with changing Q_{OA} and keeping Q_{IA} and Q_{Oil} unchanged. As a result of Q_{OA} reduction, the size of oil droplets and consequently the number of encapsulate IA cores increased (Fig. 3C). Interestingly, we also observed double-core DE droplet generation regimes by varying only Q_{IA}, as it is shown by squares graph fluctuating between one and two cores. This phenomenon happened because the inflow of the IA droplets disturbs the flow of OA phase by plugging the orifice, forcing the oil droplet cycle to synchronize so that every DE contains two cores (Fig. S7).

Although flexible in controlling the size of outer and inner droplets, the system was at the same time highly stable in generating monodisperse droplets. Fig. 3D demonstrates negligible values for coefficient of variance (C.V.) in generating both IA and oil droplets. Nevertheless, higher amount of C.V. for oil droplets could be due to lower precision of our home-made syringe pump, controlling OA flow rate, compared to other two commercial syringe pumps for IA and oil phases.

3.3. Formation of monodispersed microgels

To this end, we were able to pattern the wettability of microfluidic device and generate DE droplets (Fig. 4A). However, several requirements still remain to be met for gelation of alginate and formation of microbeads. The ideal gelation process should address the following demands: (i) divalent cations such as Ca^{2+} should be delivered to alginate solution, after its dispersion, to form a solid structure; (ii) the process of gelation needs to prevent fusion of gelling droplets and forming bigger/non-spherical/aggregated particles which can cause polydispersity and clogging the channels; (iii) the process of gelation and exploited reagents should be biocompatible; and (iv) gelation

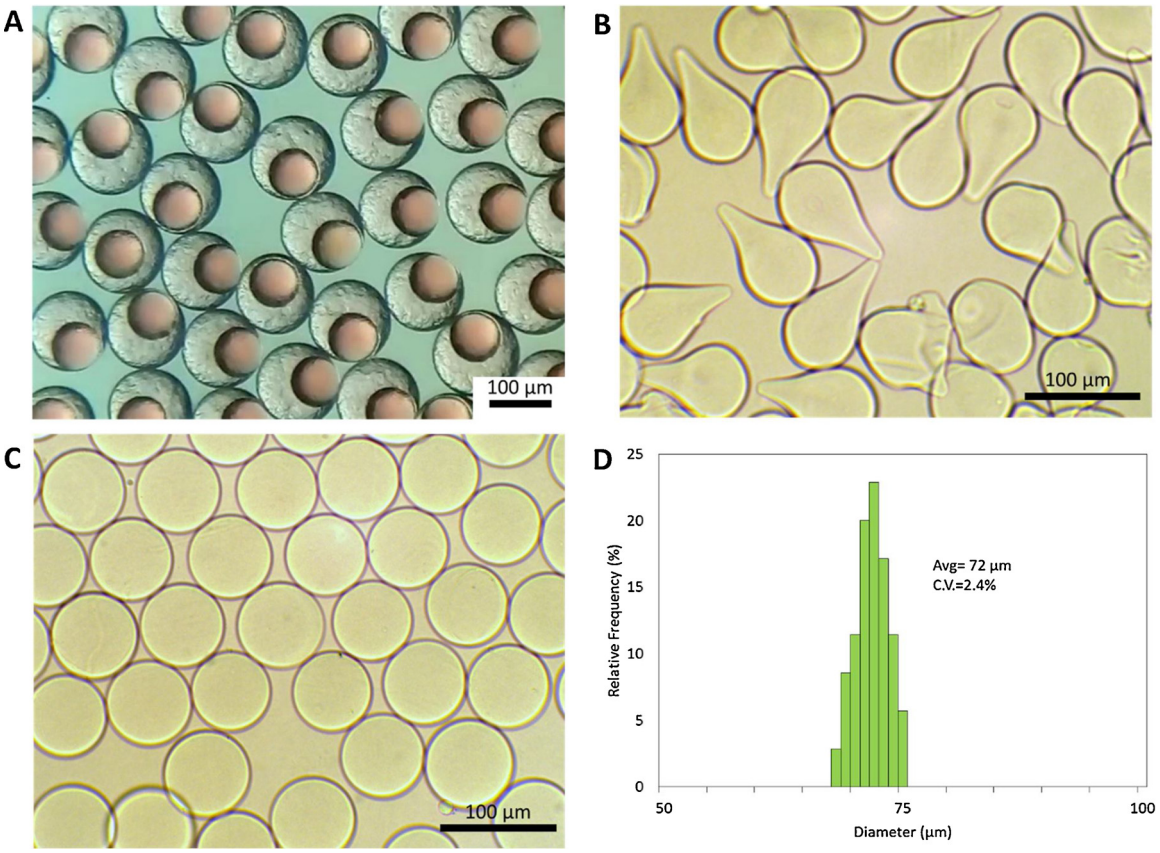

Fig. 4. Formation of alginate microgels with controlled morphology. (A) Nanoemulsion (Oil/$CaCl_2$) droplets encapsulating alginate droplets (red) which are going to be gelled. (B) Alginate microgels formed without application of $CaCl_2$ emulsified in the oil phase. Gradual crosslinking of alginate droplets upon releasing from oil droplets generated tailed-shape particles. (C) Spherical alginate microbeads formed with application of oil/$CaCl_2$ nanoemulsion as the middle phase. By adding $CaCl_2$ to the oil phase, a partial gelled layer may form on the surface of alginate droplets that prevents the shape change upon releasing from oil droplet. (D) Size distribution of spherical alginate microbeads. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

should retain the spherical morphology of the droplets. The gelation process designed in our microfluidic system meets all of the requirements listed above. External gelation of alginate droplets (in which Ca^{2+} ions are delivered from an external solution to alginate solution) in DE system offers a biocompatible and chip friendly method, as mentioned previously. The alginate solution encapsulated in the oil droplet was first exposed to $CaCl_2$ nano-drops from oil phase, and then released into outer $CaCl_2$ solution in collection bath for gelling accomplishment. Density variation in inner, middle and outer phases ($\rho_{alginate} > \rho_{water} > \rho_{oil}$) and tendency of minimizing surface energy of oil/aqueous phases interfaces induce easy release of the alginate into $CaCl_2$ bath which can be accelerated using a short centrifugation. Without application of $CaCl_2$ nano-drops emulsified in the oil phase, alginate solution gradually gels upon releasing from the oil droplets, causing tailed-shape microbeads formation (Fig. 4B). This structure of microparticles was previously reported for alginate released from DE droplets [32]. By application of oil/$CaCl_2$ nanoemulsion as the middle phase, a partial gelled shell could be formed, preventing shape change via release of the particles from the oil droplet. As a result, the issue of tailed-shape formation was resolved and spherical microbeads were harvested (Fig. 4C). Fig. S8 schematically illustrates the mechanism of alginate release from the oil droplet and formation of tailed-shape and spherical microparticles.

The narrow size distribution (C.V. = 2.4%) demonstrates capability of the method for generation of monodisperse microgels (Fig. 4D). Nevertheless, wider range of microbeads' size distribution compared to alginate droplets could be due to differential $CaCl_2$ exposure time while collecting DE droplets in gelation bath. The gelation of alginate is based on the entrapment of Ca^{2+} ion by alginate chains and forming egg-box structure that is accompanied by structure shrinkage and releasing a portion of water from the alginate solution [50]. Therefore, small variations in the size of microbeads could be stemmed from structure shrinkage due to Ca^{2+} diffusion as well as downstream swelling in buffer solution due to the replacement of Ca^{2+} with Na^{+}. Fig. S9 shows the pool of alginate microbeads suspended in DPBS solution.

$CaCl_2$ can also be added to OA phase for complete gelation of alginate droplets, possibly released early in the chip, implying that the future redesign of this microfluidic network can be used to perform gel formation and cell culture in a single chip, without any need to perform off-chip gelation, something that has been a bottleneck of droplet microfluidic technology for on-chip drug testing on microscale tissues synthesized and cultured within microfluidic systems [15].

3.4. Cell encapsulation

Biocompatibility of the external gelation method integrated with DE system made our technique an ideal candidate for cells encapsulation in monodispersed and isolated alginate microbeads (Fig. 5A). By controlling the density of dispersed cells in alginate solution, the number of cells in each microgel could be tuned (Fig. 5B). However, in high concentrations, instabilities such as early release of alginate solution from some oil droplets (due to deformation of oil/alginate interface) and polydispersity of droplet sizes (as a result of disturbance in fluid flow and droplet generation cycle) were observed. The number of encapsulated cells can also be modulated by changing the microgel size altered with variation of flow rates or geometry of microfluidic device.

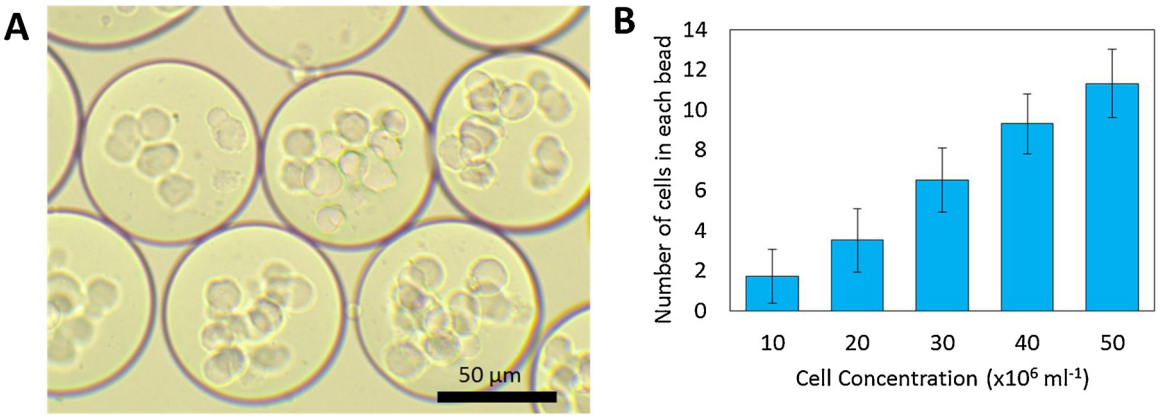

Fig. 5. Encapsulation of Human Embryonic Kidney 293 cells (HEK-293) in alginate microbeads. (A) By dispersing cells in alginate solution and generation of microbeads using the developed method in this study, cell-laden alginate microgels are formed. (B) The number of cells in each bead can be tuned by controlling the initial cell concentration dispersed in alginate solution.

Despite internal gelation methods, our approach does not need any acid or chelator reagent for fabrication of alginate microbeads. Recently, an acid-free internal gelation method was reported based on the exchanging ions between various chelators [51,52]. Exchanging the ions provides the opportunity for alginate chains to react with Ca^{2+} (one of the exchanging ions) and form egg-shaped networks, the gelled form of alginate. Despite valuable results, this method requires precise control over reagents concentration and working pH. Furthermore, the presence of chelators such as EDTA can affect the functionality of the cells. In our experiments, high level of cell viability was observed after cell encapsulation, showing biocompatibility of the fabrication method. Trypan-Blue staining (Figure S10) demonstrated that although increasing the concentration of cells decreased the viability, the percentage of viable cells still remained above 95%.

Even though alginate has many favorable properties for cell encapsulation, it lacks sequences for cell attachment such as arginine-glycine-aspartic acid (RGD) peptide as well as matrix metalloproteinase (MMP) sensitive degradation sites, suitable for cell remodeling [8]. The incapability of alginate to provide these sequences hinders good spreading of the encapsulated adherent cells (Fig. 5A). However, this feature can be exploited to promote cell-cell attachment and form spheroids using high-density cell encapsulation. Alternatively, alginate can be functionalized with RGD [28] or be mixed with other cell-favorable hydrogels [53].

4. Conclusion

Alginate hydrogel is a promising encapsulation material used in biomedical applications due to its biocompatibility and easy gelation. Despite extensive efforts, fabrication of alginate microgels using a cell and chip-friendly method is still challenging. Here, we developed a new method for fabrication of monodisperse alginate microbeads, based on external gelation in double emulsion (DE) microdroplets. For generation of DE droplets in a microfluidic device, an easy, rapid, cost-effective, and permanent approach was developed to pattern the wettability of PDMS microchannels. The whole surface modification was performed in one step, based on simultaneous plasma and PVA treatment of the desired microchannels. The method for selective PVA deposition was shown to be highly stable and the resulting microfluidic device had precise control over morphology of the DE droplets. Manipulation of flow rates in the microfluidic device tuned the size of droplets while preserving their monodispersity. The DE system was then used to fabricate alginate microbeads using a novel two-step gelation approach. Alginate, as the inner droplet of DE system, was first exposed to $CaCl_2$ emulsified in oil droplet for gelling of its surface followed by complete crosslinking upon its release to the outer $CaCl_2$ bath. This method ensures biocompatibility of microbeads formation due to the application of external gelation and rapid recovery of beads from fabrication solvents. Moreover, the developed method offers a clog-free microfluidic approach capable of generating monodisperse spherical alginate microbeads. In these structures, cells could be encapsulated with desired concentration and high level of viability, ideal for high-throughput microfluidic platforms in tissue engineering and drug discovery applications. The facile and robust DE generation strategy proposed here is not limited to the fabrication of alginate microbeads by external gelation but it can also provide opportunities for reducing the clogging and toxicity limitations of other microbeads fabrication methods. This method can be further used in other high-throughput droplet-based biological assays such as digitalized fluorescent activated sorting and polymerase chain reaction (PCR) [54,55].

Notes

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2019.04.100.

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