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One-step generation of cell-laden microgels using double emulsion drops with a sacrificial ultra-thin oil shell†

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Cell-laden microgels with highly uniform sizes have significant potential in tissue engineering and cell therapy due to their capability to provide a physiologically relevant three-dimensional (3D) microenvironment for living cells. In this work, we present a simple and efficient microfluidic approach to produce monodisperse cell-laden microgels through the use of double emulsion drops with an ultra-thin oil shell as the sacrificial template. Specifically, the thin oil shell in double emulsion spontaneously dewets upon polymerization of the innermost precursor drop and subsequent transfer into an aqueous solution, resulting in direct dispersion of microgels in the aqueous phase. Compared to conventional single emulsion-based techniques for cell encapsulation, this one-step approach prevents prolonged exposure of cells to the oil phase, leading to high-throughput cell encapsulation in microgels without compromising the cell viability. Moreover, this approach allows us to culture cells within a 3D microgel which mimics the extracellular matrix, thus enabling long-term cell functionality. This microfluidic technique represents a significant step forward in high-throughput cell microencapsulation technology and offers a potentially viable option to produce cell-laden microgels for widespread applications in tissue engineering and cell therapies.

Introduction

Hydrogels consisting of a biocompatible and biodegradable polymeric matrix provide a physiologically relevant three-dimensional (3D) microenvironment for living cells.[1–5] However, cells embedded within macroscopic hydrogels suffer from restricted intracellular communications and nutrient exchange due to the limited diffusion rate and distance of extracellular molecules through the crosslinked network.[6] In contrast, microgels on the microscopic scale are more suitable carriers for cell encapsulation and 3D culture, as they ease mass transport and facilitate higher control over the environmental cues of extracellular matrices.[7–9] Thus, high-throughput encapsulation of cells in microgels can potentially enable major advances in tissue engineering and cell therapy strategies.[1,7] Such microgels loaded with living cells can serve as building blocks that allow assembly into complex tissue mimics,[10] or can act as carriers for controlled delivery in cell therapy.[11] However, it is critical to control both the size and size distribution of the delivery vehicles as they can affect bioavailability; moreover, they also determine the behavior of the encapsulated cells.[5,12] This demands techniques to produce microgels that are both highly controlled in size and structure, and in which cells can be easily encapsulated while retaining very high viability.

Several microfabrication techniques to produce cell-laden microgels have been proposed. These approaches make use of photolithography,[13] micromolding[9,14] and centrifuge-based drop makers,[15] providing many advantages such as scalability and precise control over particle size and shape. However, these conventional techniques are limited by the inherent nature of batch processing, resulting in low throughput. Recent advances in microfluidic techniques enable precise control of immiscible multiphase flows, providing continuous and rapid production of monodisperse microgels with controllable sizes. For example, water-in-oil (w/o) emulsion drops that are formed using a T-junction[16] or through flow-focusing[17] can be used as templates for producing monodisperse microgels with a variety of polymerization schemes.[18–20] However, this approach is not efficient due to the use of a continuous oil phase, which can significantly decrease the viability of immobilized cells due to prolonged exposure to the oil and surfactants that can be potentially cytotoxic.[8,21] In addition, an extra washing step is needed to

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break the emulsion and transfer the resulting microgel particles into an aqueous medium for use, which makes this approach time- and labor-consuming. Alternatively, water-in-water emulsion drops, an aqueous two-phase system, can be used as a template to produce microgels in an aqueous medium, avoiding the extra washing steps.[22,23] However, this system is limited to specific combinations of two immiscible aqueous solutes, such as dextran and polyethylene glycol, which precludes the widespread use of this technique for cell encapsulation. Thus, it still remains a challenge to produce monodisperse microgels that can effectively encapsulate cells while retaining their viability, and new techniques to accomplish this are required.

In this work, we report a simple and efficient microfluidic approach to produce monodisperse microgels by utilizing an ultra-thin oil shell of double emulsion drops as a sacrificial template. Using a glass capillary device, we form a coaxial flow of an aqueous prepolymer solution surrounded by an oil phase that is subsequently emulsified into a continuous aqueous phase, resulting in monodisperse double emulsion drops, as shown schematically in Fig. 1a. Upon UV exposure, the innermost drops composed of the prepolymer solution are selectively solidified, and the drops are then immediately transferred to an aqueous solution (Fig. 1b). The surrounding thin oil shell in double emulsion spontaneously dewets upon polymerization of the innermost drop and subsequent transfer into an aqueous solution, thereby separating the oil phase from the microgels and directly dispersing them into the aqueous solution. We further demonstrate that this one-step approach can be extended to achieve scalable production of cell-laden microgels while retaining high cell viability by preventing prolonged exposure of cells to the oil phase. By utilizing a biocompatible and biodegradable polymer as the extracellular matrix, we provide the encapsulated cells with a physiologically relevant 3D microenvironment and enable long-term cell functionality within the gel matrix.

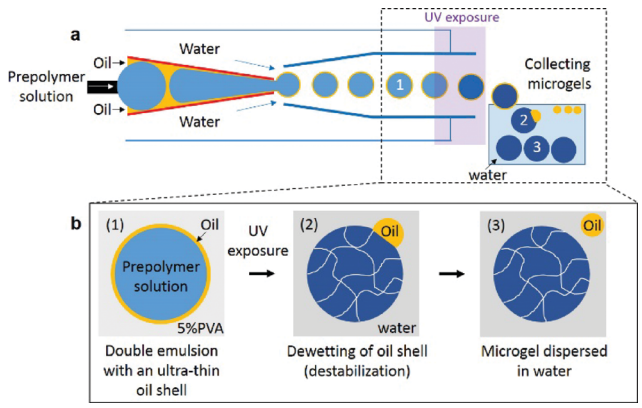

Fig. 1 One-step production of microgels through the use of double emulsion drops with an ultra-thin oil layer. (a) Schematic illustration showing a glass capillary microfluidic device for the preparation of double emulsion drops. The innermost drops are solidified to form microgels upon UV exposure. (b) Schematic illustration showing the detailed procedure to form solid microgels from double emulsion drops through a dewetting process of the oil layer.

Results and discussion

Formation of double emulsion drops with an ultra-thin sacrificial oil shell

To make double emulsion drops with an ultra-thin sacrificial oil layer, we use a glass capillary microfluidic device composed of two tapered circular capillaries inserted into a square capillary.[24] We use *n*-octadecyltrichlorosilane to make the circular injection capillary wall hydrophobic. In addition, a small tapered capillary is inserted into the injection capillary to facilitate simultaneous injection of two immiscible fluids. Another circular capillary is inserted into the square capillary at the other side to confine the flow near the injection tip, thereby increasing the flow velocity; this is treated with PEG-silane to make the capillary wall hydrophilic. The assembly of the capillary microfluidic device is illustrated schematically in Fig. 1a.

An aqueous prepolymer solution is injected through the small tapered capillary to form the innermost drop, while an oil phase is injected through the injection capillary. The co-injection of these two immiscible fluids leads to a coaxial flow that consists of an ultra-thin oil layer surrounding the innermost fluid of the prepolymer phase due to the strong affinity of the oil phase to the hydrophobic wall of the injection capillary. An additional aqueous phase is injected through the interstices of the square and injection capillaries from the same side with the injection capillary. The coaxial flow from the injection capillary is emulsified in a dripping regime by the continuous aqueous phase at the exit of the injection capillary, resulting in the formation of monodisperse double emulsion drops with an ultra-thin oil layer, as shown in Fig. 1b-1. Upon exposure to UV illumination, the innermost drops, containing the prepolymer solution, are selectively solidified, forming microgels in the core as shown in Fig. 1b-2. Collecting these microgels in an aqueous solution without any surfactants causes the ultra-thin oil layer to dewet from the surface of the microgels, resulting in the formation of cell-laden microgels directly in water, without the additional washing step, as shown in Fig. 1b-3.

Production of monodisperse microgels through dewetting of the oil shell

To produce the microgels, we prepare monodisperse double emulsion drops with an ultra-thin oil layer using a capillary microfluidic device operating in the dripping regime,[25] as shown in the optical image in Fig. 2a. To accomplish this, we use an aqueous solution of 10% polyethyleneglycol diacrylate (PEG-DA) for the innermost prepolymer phase, mineral oil with 0.5% Span80 as the middle oil phase and an aqueous solution of 5% PVA as the continuous phase. The stream of double emulsion drops is exposed to UV illumination; this leads to selective polymerization of the innermost prepolymer drops. To visualize how the microgels are formed from the

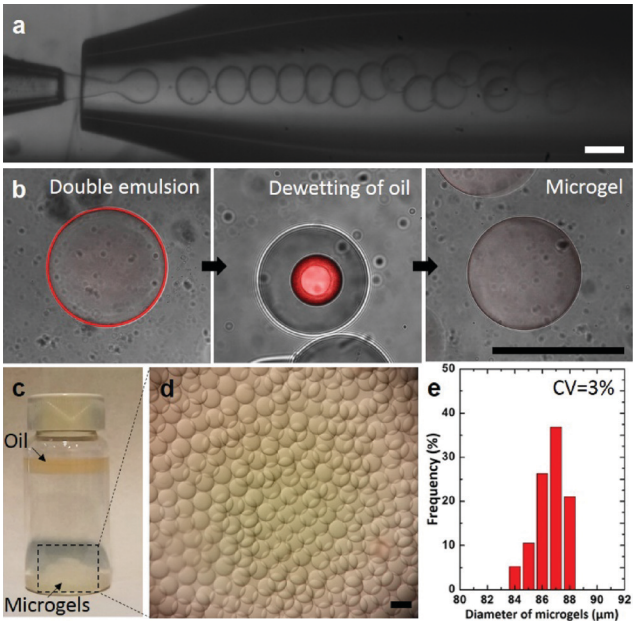

Fig. 2 Monodisperse microgels produced by a glass capillary microfluidic device. (a) Optical image showing the continuous formation of double emulsion drops in a dripping mode. (b) A series of confocal images showing the dynamic behavior of the oil layer as the polymerized drops are transferred in an aqueous solution (DI water). The estimated thickness of the oil shell is approximately 1 μm using image analysis. (c–d) Optical images showing the resulting microgels on the bottom surface and the oil layer collected on the top surface after the dewetting process. (e) Size distribution of the resulting microgels. All scale bars represent 100 μm.

double emulsions, we label the ultra-thin oil layer with an oil-soluble fluorescent dye (Nile red). Close examination of the microgels upon collection in an aqueous solution without any surfactants reveals that the ultra-thin oil layer completely engulfs the microgels. However, after 1 min, the thin oil layer starts to gradually dewet from the surface of the microgels, leading to the segregation of oil drops on the surface of the microgels and ultimately to the separation of the oil drops from the microgels, as shown in Fig. 2b. We attribute the dewetting process to the significant change in the interfacial tension among the fluids comprising the emulsions when they are transferred into an aqueous solution in the absence of surfactants. Specifically, the lack of surfactants causes the oil layer in double emulsions to destabilize, making it separate from the innermost phase and thus forming oil-free microgels in a consistent manner. The separated oil drops immediately migrate to the upper region of the vial due to buoyancy ($\rho_{\text{mineral oil}}$ = 0.840 g mL^{-1} and $\rho_{10\%\ \text{PEG-DA aq.}}$ = 1.01 g mL^{-1}), as shown in Fig. 2c; this facilitates the collection of microgels, as shown in Fig. 2d. The resulting microgels are monodisperse with a coefficient of variation of 3%, as shown by the size distribution curve in Fig. 2e.

Encapsulation of mammalian cells with high viability

Compared to conventional single-emulsion techniques for microgel preparation, this thin-shell double emulsion approach avoids the time- and labor-consuming washing step and achieves direct transfer of the resulting microgels from the oil phase into the aqueous phase; this leads to the one-step production of microgels in a continuous manner. Importantly, this approach significantly reduces the residence time of the microgels within the oil phase from hours to minutes. This improves the viability of encapsulated cells by avoiding prolonged exposure of the cells to the potentially harmful oil and surfactants.[21] To demonstrate this, we compare the viability of encapsulated cells using the conventional single emulsion technique with the current one-step thin-shell double emulsion one. We encapsulate living Madin–Darby canine kidney epithelial (MDCK) cells using a biocompatible polymer (polyethyleneglycol diacrylate, PEG-DA, or gelatin methacrylate, GelMA) as the microgel material. To prepare these microgels, we first dissolve the hydrogel precursor and the photo-initiator (Irgacure 2959) into cell culture medium (*i.e.* DMEM). Then, we disperse the cells into this solution with a concentration of 1 × 10^6 cells per ml. For cell encapsulation, we use mineral oil containing 0.5% Span 80 surfactants or fluorinated oil (HFE 7500) containing 0.5% Krytox-PEG-Krytox surfactants as the middle oil phase. An aqueous solution of 5% PVA is used as the continuous phase to form double emulsion drops. A schematic illustration of the cell encapsulation procedure based on double emulsions with a sacrificial oil shell is shown in Fig. 3a. We successfully demonstrate the feasibility of this technique for cell encapsulation as shown in the optical image in Fig. 3b. Upon UV illumination ($\lambda \sim$ 365 nm for 2 s), cells are immobilized as the gel matrix is solidified. Collection of the cell-loaded microgels in an aqueous solution of cell culture media (DMEM) leads to spontaneous dewetting of the oil phase on the surface of microgels, allowing autonomous transfer of microgels into the aqueous phase. The resulting microgels contain multiple cells and have an average diameter of 244 μm, as shown in the fluorescence microscopy images in Fig. 3c and d. The number of encapsulated cells per microgel and the size of microgels can be modulated by varying the concentration of the cell suspension, the nozzle size of the capillary, and the fluid flow rates.[8,26] We observe reduced monodispersity of the cell-laden microgels (see ESI,† Fig. S1) compared to the cell-free ones; this can be attributed to the inhomogeneity of the cell suspension, leading to variability in the drop size during emulsification and subsequent break-up.

The rapid dewetting allows spontaneous transfer of the resulting cell-laden microgels into cell-culture media, therefore avoiding extensive exposure of living cells to the oil phase which can depress the viability and metabolic activity of the cells, and providing unperturbed nutrient exchange through the hydrogel layer. Indeed, we observe that cells encapsulated in microgels using the one-step double emulsion technique exhibit survival rates comparable to those of cells cultured on a tissue culture plate (polystyrene) as evidenced by the Live/Dead staining assay performed after 3 h of microfluidic cell encapsulation (Fig. 3c and d). In contrast, cell viability within microgels from the single emulsion approach

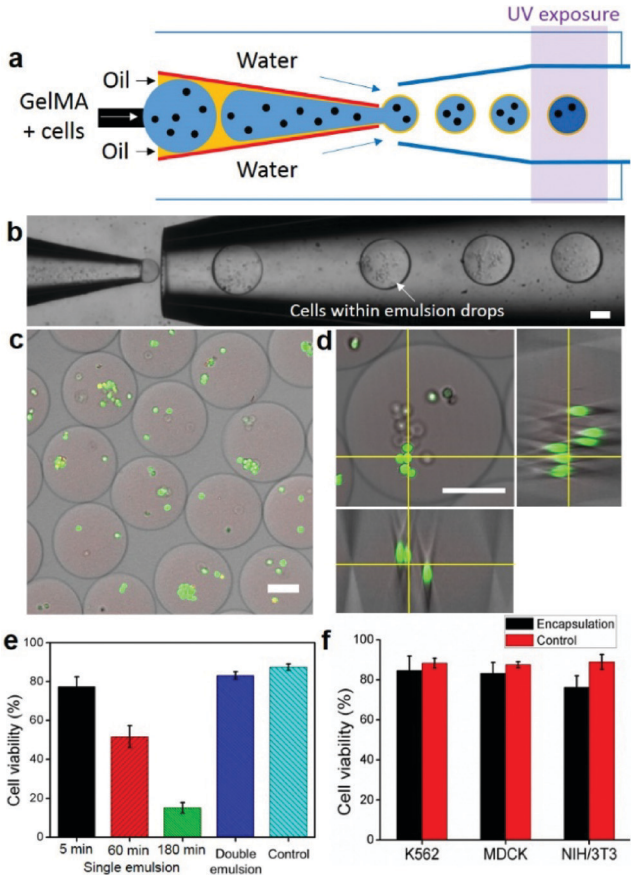

Fig. 3 Production of cell-laden microgels. (a) Schematic illustration showing the encapsulation of living mammalian cells using the thin-shell double emulsion drops formed with a photo-crosslinkable polymer. (b) Optical image showing cell encapsulation within double emulsion drops. (c) Representative fluorescence microscopy image showing cells embedded within microgels. Cells were stained by calcein-AM (green-fluorescent dye for live cells) and ethidium homodimer (red-fluorescent dye for dead cells). (d) Representative fluorescence microscopy image of a single mcirogel containing multiple cells. Orthogonal view showing the three-dimensional cell distribution within the microgels. (e) Survival rates of MDCK cells encapsulated in microgels prepared by the conventional two-step single emulsion approach in comparison with cells encapsulated by the proposed one-step thin shell double emulsion approach. The effect of the two-step approach on cell viability was evaluated by incubating the encapsulated cells within the emulsion containing mineral oil and Span80 for different periods of time (5, 60 and 180 min), and then determining the survival rates of the cells. The fraction of cell viability was determined from LIVE/DEAD staining. The control group is cells that have been dispersed in the polymer precursor solution followed by 2D culture on a tissue culture plate. (f) Survival rates of different cell types after microfluidic encapsulation using the thin-shell double emulsion method using HEF and Krytox-PEG-Krytox surfactants as the oil phase. All scale bars represent 100 μm.

significantly decreases as the residence time of the cells within the oil phase increases. As shown in Fig. 3e, long-term exposure (3 h) of cell-laden microgels to the oil phase (herein mineral oil containing 0.5% Span 80 surfactants) results in less than 20% survival rate of the cells. This cytotoxicity of the conventional method can be related to i) the prolonged exposure of encapsulated cells to the surfactants which could potentially lead to rupture of the cell membrane,[21] ii) the cytotoxicity of the photo-initiator,[27,28] and the limited nutrient/gas exchange in the oil phase. Further, we use fluorinated oil (HFE 7500) containing 0.5% Krytox-PEG-Krytox surfactants as the middle oil phase instead of mineral oil and Span80; this could improve the cytocompatibility of the oil phase because of the negligible solubility of fluorinated oil in water (3 ppm) and its high gas permeability.[29] We observe that all three cell types tested (*i.e.* myelogenous leukemia cells (K562), MDCK cells and NIH/3T3 fibroblast cells) exhibit desirable survival rates after encapsulation (Fig. 3f), confirming the biocompatibility of our approach for cell encapsulation for a variety of cell types. These results indicate that the conventional single-emulsion approach is not suitable for scale-up production of cell-laden microgels since cells have to be trapped within the oil phase for a period of time to collect enough samples. In contrast, the double emulsion method enables continuous cell encapsulation without compromising the cell viability, which can serve as a cost-effective tool for scalable 3D cell encapsulation.

Three-dimensional (3D) cell culture within microgels

We further demonstrate the biocompatibility of this encapsulation technique by culturing the mammalian cells within the three-dimensional microenvironment. We encapsulate and subsequently culture MDCK and NIH/3T3 cells (both are anchorage-dependent cells) using biodegradable GelMA microgels. Gelatin matrices allow cells to adhere and proliferate three-dimensionally due to the presence of a cell-attachment site (*i.e.* Arg–Gly–Asp, RGD sequence) in gelatin molecules,[30] and maintain a healthy and viable state to proceed with their normal functionality. Indeed, we observe continuous growth of both cell types within the gelatin matrices (Fig. 4). MDCK cells reproduce and aggregate with each other to form stratified cell spheroids after 4 days (Fig. 4a and b). The size of the spheroidal cellular constructs increases gradually (Fig. 4c), which eventually leads to the formation of a specific architecture resembling simple epithelial tissues after 7 days (see ESI,† Fig. S2). Such a cyst structure with a multi-cell thick shell and a central lumen is representative of the 3D culture of MDCK cells in a collagen matrix,[31] confirming the biocompatibility of our approach.

Unlike epithelial MDCK cells that form coherent, densely packed cellular organization, NIH/3T3 fibroblasts exhibit a completely different behavior after being encapsulated into GelMA microgels. Remarkably, we observe cell attachment and spreading within the 3D matrix three hours after encapsulation, as evidenced by the penetration of pseudopodia throughout the matrix (Fig. 4e and f). The majority of encapsulated cells egress out from the internal space of the microgels and attach to the surface of the gels after 4 days. Interestingly, the GelMA microgels severely deform from their original spherical shape and considerably reduce the size of the microgels (Fig. 4d), most likely due to the compressive forces exerted by cell adhesion to the hydrogel matrices.

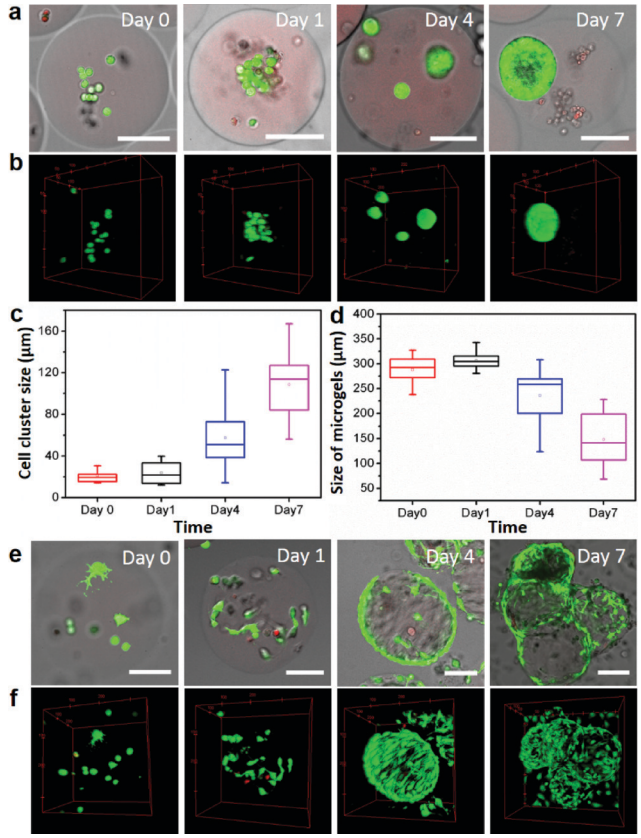

Fig. 4 3D cell culture in GelMA microgels. (a) Representative fluorescence microscopy images of the cross-section of the cell-laden microgels and (b) corresponding 3D reconstruction showing the growth of MDCK cells embedded within the microgels at different time points during 3D culture. Cells were stained with calcein (green-fluorescent dye for live cells) and ethidium homodimer (red-fluorescent dye for dead cells). (c) The size of the cell clusters (characterized by the diameter of the cell spheroids) formed within the GelMA microgels at different time points during 3D cell culture. (d) The diameter of the cell-laden microgels (characterized by the diameter of the microgels) at different time points during 3D cell culture. (e) Representative fluorescence microscopy images of the cross-section of the cell-laden microgels and (f) corresponding 3D reconstruction showing NIH/3T3 fibroblast cells embedded within the microgels at different time points during 3D culture. All scale bars represent 100 μm.

Moreover, the growth of fibroblasts eventually bridges and aggregates the neighboring microgels. These results confirm that our approach could provide an efficient platform to create these cell-laden microgels that can serve not only as a microscopic platform for the study of long-term cell functionality in a 3D microenvironment, but also as building blocks for the bottom-up assembly of complex cellularized constructs with a tissue-specific architecture.

Experimental

Preparation of double emulsions with a sacrificial ultra-thin oil shell

To produce double emulsion drops, we inject an 8–10% aqueous solution of polyethyleneglycol diacrylate (PEG-DA, M_n 700, Sigma-Aldrich) and gelatin-methacrylate (GelMA) through a small tapered capillary with a typical flow rate of 1000 μL hr^{-1}. A mineral oil (Sigma-Aldrich) containing 0.5% Span80 (Sigma-Aldrich) as a non-ionic surfactant or HFE-7500 (3 M) containing 0.5% Krytox-PEG-Krytox (Ran Biotechnologies, Inc.) is simultaneously supplied through the injection capillary with a typical flow rate of 1000 μL hr^{-1}. An aqueous solution of 5% poly(vinyl alcohol) (PVA) (M_w 13 000–23 000, Sigma-Aldrich) is injected through the interstices of the square and collection capillaries with a typical flow rate of 15 000 μL hr^{-1}. These rates ensure that drop formation is restricted to the dripping regime.

Preparation of the microfluidic device and drop generation

We use a glass capillary microfluidic device to produce double emulsion drops with an ultra-thin oil layer. We prepare an injection capillary by tapering a 560 μm inner diameter cylindrical glass capillary (1B100-6, World Precision Instruments, Inc.) to 50 μm inner diameter; to make the inner wall hydrophobic or fluorophilic, we treat it with *n*-octadecyltrimethoxyl silane (Aldrich) or heptadecafluoro-1,1,2,2 tetrahydrodecyl trichlorosilane (Gelest, Inc.), respectively, for 10 minutes and subsequently wash it with ethanol. We insert the injection capillary into a square capillary (AIT Glass) whose inner width (1.05 mm) is slightly larger than that of the outer diameter of the injection capillary (1 mm). Next, we prepare a small tapered glass capillary (10 μm inner diameter) by heating and pulling a cylindrical capillary by hand using a gas torch; this capillary is inserted into the injection capillary for simultaneous injection of two immiscible fluids. Finally, a cylindrical collection capillary is inserted into the square capillary from the other end; we also treat this collection capillary with 2-[methoxy(polyethyleneoxy)-propyl] trimethoxy silane (Gelest, Inc.) to make the capillary wall hydrophilic. During drop generation, the volumetric flow rate is controlled by syringe pumps (Harvard Apparatus) and the production of emulsion drops is observed using an inverted microscope equipped with a high-speed camera (Phantom V9.0). The experimental setup is shown in Fig. S3.†

Cell encapsulation and 3D culture

Myelogenous leukemia cells (K562), Madin–Darby canine kidney epithelial (MDCK) cells and NIH/3T3 fibroblast cells are obtained from ATCC®, and are cultured for 6 days in proliferation medium (Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich), supplemented with 10% v/v fetal bovine serum (FBS, Gibco), at 37 °C, 95% relative humidity and 5% CO_2. The medium is refreshed every three days of culture. At confluence, cells are washed twice with PBS, detached using trypsin/EDTA (0.25% w/v trypsin/0.02% EDTA) for 5 min and resuspended in cell culture media. GelMA and the photoinitiator (Irgacure 2959) are dissolved in DMEM followed by sterilization and filtration. All devices are sterilized prior to use by exposure to ultraviolet illumination ($\lambda \sim 254$ nm) for 60 min. Cell-laden microgels are prepared by encapsulating

the cells suspended in DMEM containing 10% w/v of PEG-DA or GelMA and 1% w/v photo-initiator in double emulsion drops. Then these double emulsion drops are *in situ* photo-polymerized in UV for 2 s to form cell-laden microgels. The cell-laden microgels are collected using a cell strainer followed by re-dispersion in cell culture media. The medium is refreshed every three days of culture. Cell survival is determined using a LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes). To this end, the gels are washed in sterile PBS for 30 min at 37 °C prior to incubation for 30 min at room temperature with 2 mM calcein-AM (red-fluorescent dye for dead cells) and 4 mM ethidium homodimer (green-fluorescent dye for live cells) in PBS solution. After incubation, the gels are rinsed in PBS and examined using a Leica SP5 confocal laser scanning microscope with a 40× water-immersion objective lens.

Conclusions

In this paper, we present a one-step microfluidic approach to produce monodisperse microgels by utilizing double emulsion drops with an ultra-thin oil shell as a sacrificial template. This approach allows the production of microgels in a simple and efficient manner, avoiding the extra washing procedure required for conventional single emulsion-based approaches. In addition, we demonstrate the advantages of this method by encapsulating and culturing living mammalian cells in three-dimensional microgel matrices; we achieve high cell viability by avoiding potential cytotoxicity from prolonged exposure to oil and surfactants. We anticipate that the cell-laden microgels with tunable sizes and compositions fabricated using this microfluidic platform are not limited to UV-induced polymerization but can also be formed using other polymerization schemes such as chemical diffusion and temperature-induced solidification. Moreover, this approach allows us to manipulate cells at the microscopic scale based on microfluidics and to observe long-term cell functionality within the microgel matrices. This microfluidic technique represents a significant step forward in high-throughput cell microencapsulation technology and offers a potentially viable option to produce cell-laden microgels for widespread applications in tissue engineering and cell therapy.

Acknowledgements

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