Fabrication of uniform multi-compartment particles using microfluidic electrospray technology for cell co-culture studies

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Fabrication of uniform multi-compartment particles using microfludic electrospray technology for cell co-culture studies[a)]

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In this work, we demonstrate a robust and reliable approach to fabricate multi-compartment particles for cell co-culture studies. By taking advantage of the laminar flow within our microfluidic nozzle, multiple parallel streams of liquids flow towards the nozzle without significant mixing. Afterwards, the multiple parallel streams merge into a single stream, which is sprayed into air, forming monodisperse droplets under an electric field with a high field strength. The resultant multi-compartment droplets are subsequently cross-linked in a calcium chloride solution to form calcium alginate micro-particles with multiple compartments. Each compartment of the particles can be used for encapsulating different types of cells or biological cell factors. These hydrogel particles with cross-linked alginate chains show similarity in the physical and mechanical environment as the extracellular matrix of biological cells. Thus, the multi-compartment particles provide a promising platform for cell studies and co-culture of different cells. In our study, cells are encapsulated in the multi-compartment particles and the viability of cells is quantified using a fluorescence microscope after the cells are stained for a live/dead assay. The high cell viability after encapsulation indicates the cytocompatibility and feasibility of our technique. Our multi-compartment particles have great potential as a platform for studying cell-cell interactions as well as interactions of cells with extracellular factors.
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I. INTRODUCTION

Co-culture of cells is of great importance for studying interaction of cells. In some co-culture studies, cells of different types are seeded in the same mixture and the separation distance is sufficiently small for them to touch each other, while in other cases, different cells are physically separated.[1,2] In typical non-contact cell co-culture system, different cell types are cultured in the same chambers while remaining physically separated by the cell culture insert.[3,4] During the co-culture process, the semi-permeable membrane of the cell culture insert allows the transportation of nutrients and cell factors while inhibiting the contact of different cell types. However, it is often difficult to create a microenvironment with spatial or temporal changes in a two-dimensional (2-D) adherent co-culture system. Recently, the emergence of microfluidic device has enabled the manipulation of extracellular microenvironment with controlled flows. In microfluidic devices, compartmentalized chambers and channels are built by combining several layers of substrates prepared using techniques such as soft-lithography, laser engraving, and photolithography.[5–8] The membranes separating the connected channels between the different chambers or flow channels allow the perfusion of nutrients and cell factors.[8,9] By

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culturing cells of different types in the chambers and flowing nutrients in the channels, long-term study of the interaction and growth of cells can be carried out.[7,8] Co-culture devices using either culture dish or microfluidic chambers provide good extracellular environment for the growth of cells and has enabled the study of cell-cell interaction and cell growth. However, cells in complex and three-dimensional tissues or organs behave differently from cells in two dimensional culture dish or microfluidic chambers. One important difference between these artificial microenvironments and the natural environment is the absence of a supporting extracellular matrix (ECM) around cells; this may significantly influence the cell behaviors as the biological relevance between cells and ECM is precluded.[9–11] Due to the similarity in mechanical properties between hydrogels and extra cellular matrix, hydrogels with cells embedded inside are commonly used to simulate the ECM structure of *in vivo* tissue in artificial cell culture system.[11–15] However, the size and the shape of these hydrogel spheroids are often hard to be precisely controlled.[11]

Multi-compartment particles are particles with distinct segments, each of which can have different compositions and properties. Several approaches have been used to fabricate micron-sized multi-compartment particles; these include microfluidics. With the microfluidic approach, monodisperse water-oil emulsions are used as templates, which are subsequently crosslinked to form the micro-particles.[16] For instance, to prepare Janus particles, which are particles with two hemispheres of different compositions, two parallel stream of distinct dispersed phases are first generated in the micro-channels. Then the two streams emerge as a combined jet in the continuous phase without significant mixing. Eventually, the jet breaks up into uniform micro-droplets because of the Rayleigh-Plateau instability.[17] Afterwards, the Janus particles are formed following photo-polymerization induced by ultraviolet light. This microfluidic method enables the fabrication of Janus particles at a high production rate and with a narrow size distribution. However, the oil-based continuous phase can remain attached to the final particles and be difficult to be washed away completely. This limits the use of these particles in biological applications. To overcome this limitation, we propose to combine the microfluidic approach with electrospray, which takes advantage of electrical charging to control the size of droplets, and to fabricate these multi-compartment particles. In the nozzles with microfluidic channels, dispersed phases with different ingredients are injected into multiple parallel channels, where these laminar streams combine to a single one upon entering a larger nozzle. Unlike the microfluidic approach, which uses a shear force alone to break the jet into fine droplets, we apply electrostatic forces to break the jet into uniform droplets. Our microfluidic electrospray approach for fabricating multi-compartment particles does not involve any oil phase, thus significantly simplifying the fabrication procedures. We demonstrate that with our approach, multi-compartment particles can be easily generated with high reproducibility.

In this work, we propose to use multi-compartment particles, which are fabricated by microfluidic electrospray with shape and size precisely controlled, to simulate the micro-environments in biological cells for co-culture studies. These particles with multiple compartments are made of alginate hydrogels with a porous structure similar to that of the extracellular matrix. Alginic acid is chosen as the matrix material for its excellent biocompatibility among many kinds of natural and synthetic polymers.[18,19] Different cell types or biological cell factors can be encapsulated inside the compartments of the particles but remain separated from each other; the semi-permeable nature of the hydrogel allows the transport of the nutrients and cell factors throughout the particles. This make the particles a promising three-dimensional platform for studying interactions between different cell types.

II. EXPERIMENTAL DETAILS

A. Material preparation

2 w/w% sodium alginate (Aladdin Chemistry Co., Ltd, China) dissolved in PBS buffer is used as the precursor solution. After sterilization by autoclaving at 121 °C for 20 min, the precursor solution is then mixed with different ingredients, such as dye molecules, cells or cell factors, to prepare the dispersed phases, which eventually fill the different compartments of the final

particles. Dye molecules are introduced to facilitate visualization of the compartments. For the cell encapsulation experiments, 3T3 fibroblast cells are mixed with the precursor solution to form a cell suspension with cell density of $1*10^6$ cells/ml. 3 w/w% calcium chloride (Wing Hing Chemical Co., Ltd., Hong Kong) solution is added to a collection bath for collecting the micro-droplets. After the micro-droplets with multiple compartments are dropped into the bath containing calcium chloride solution, the calcium ions (Ca^{2+}) cross-link the alginate chains and alginate hydrogel particles with multi-compartment morphology are formed, as shown in Fig. 1(c).

B. Electrospray setup

The dispersed phases are driven by syringe pumps (Model Lsp01-2A, Baoding Longer Precision Pump Co., Ltd.). The different dispersed phases are first pumped through different metal needles and then merge into one single stream in a larger metal needle. High-strength electric field is formed between the metal nozzle and a ground circular electrode connected to a high voltage power supply, as shown in Fig. 1(a). With increasing strength of the electric field, the dispersed liquid is gradually ionized and forms a tapered tip driven by the electrostatic force. Afterwards, the jet with the tapered tip shape breaks up into micro-droplets in the high-strength electric field, as shown in Fig. 1(b). The process of droplets formation is captured using a high speed camera (Phantom v9.1) equipped with a zoom lens (Nikon AFS DX 18-55 MM); an additional light source is added to provide the illumination needed, as demonstrated in Figure 1(a).

C. Cell culture and cells viability

3T3 fibroblast cells were cultured at a temperature of 37 °C in culture plates containing a culture medium which is made up of High Glucose Dulbecco's Modified Eagle Medium (DMEM-HG), 10% Fetal Bovine Serum (FBS) and 1% of Penicillin/Streptomycin (10 000 units/ml penicillin and 10 000 μg/ml Streptomycin). Cells inside the multi-compartment particles are stained with calcein-AM/ethidium homodimer-1 Live/Dead assay (Life technologies, Hong Kong) for 1 h before the viability of the cells is tested under a fluorescence microscope (Model Eclipse TE2000-U, Nikon).

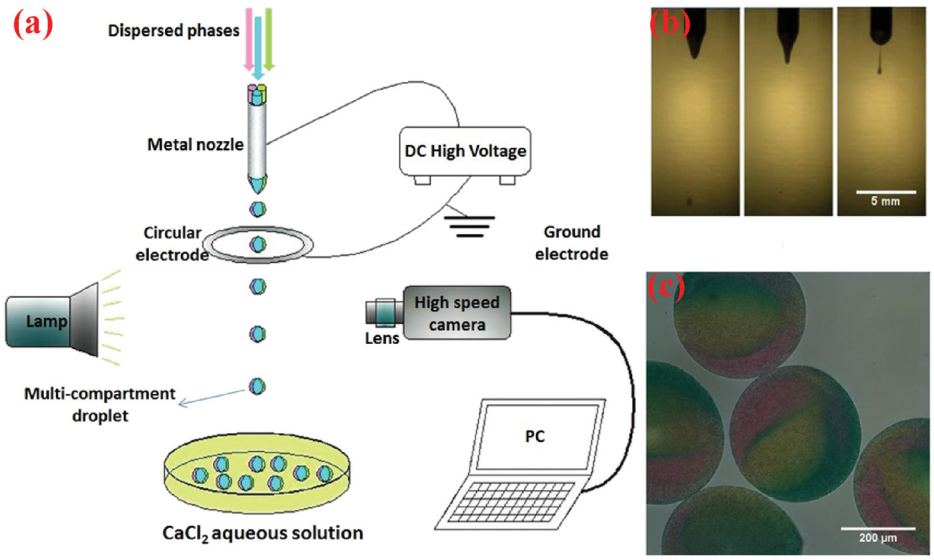

FIG. 1. (a) Sketch of the experimental setup; (b) images of the droplet formation captured by a high speed camera; (c) optical microscope image of three-compartment particles.

III. RESULTS AND DISCUSSIONS

A. Droplet formation and size distribution

The size of the droplets formed by electrospray depends critically on the strength of the applied electric field,[20] as shown by Figures 2(a)–2(f). Generally, with an increase in the electric field strength, the size of the droplets formed decreases (Figure 2(g)). When no electric field is applied between the nozzle and the circular electrode, droplet formation is purely dominated by interplay of surface tension and gravity. The droplets formed have a size that is correlated to the diameter of nozzle (Figure 2(a)). With an increase in the electric field strength, fluid dispensed through the nozzle is stretched by the increased electrostatic force and forms a tapered jet. Smaller droplets are formed as the jet breaks up at the tip (Figures 2(b)–2(d)). When the electrostatic force becomes comparable with the gravitational force, we can observe an unstable fluctuating jet; this leads to polydisperse droplets, as shown in Figure 2(e). During the jet breakup process, satellite droplets are formed together with the larger parent droplets (Figure 2(h)); this broadens the size-distribution of the resultant droplets. When the strength of the electric field is further increased, the pulling force against surface tension is dominated by the electrostatic force rather than gravity. Consequently, a stable tapered jet is observed and relatively monodisperse droplets are formed (Figure 2(f)). A typical polydispersity of the resultant

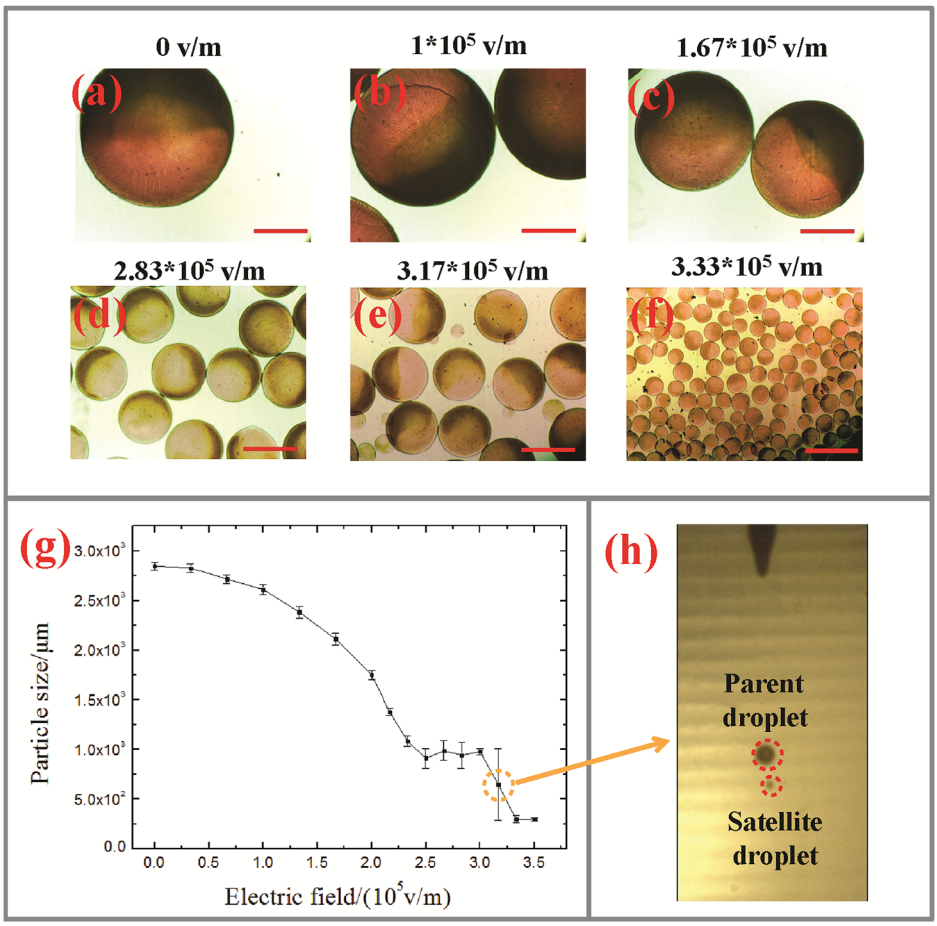

FIG. 2. Optical images of Janus particles formed by microfluidic electrospray with the electric field strength of (a) 0 V/m, (b) 1×10^5 V/m, (c) 1.67×10^5 V/m, (d) 2.83×10^5 V/m, (e) 3.17×10^5 V/m, (f) 3.33×10^5 V/m, respectively. The flow rate of the fluid is constant (10 ml/h) and the scale bar is 1 mm; (g) a plot of the particle size as a function of the strength of the electric field; (h) an image of the droplet formation process captured by a high speed camera. In the microfluidic electrospray process, the flow rate is 10 ml/h and the electric field strength is 3.17×10^5 v/m.

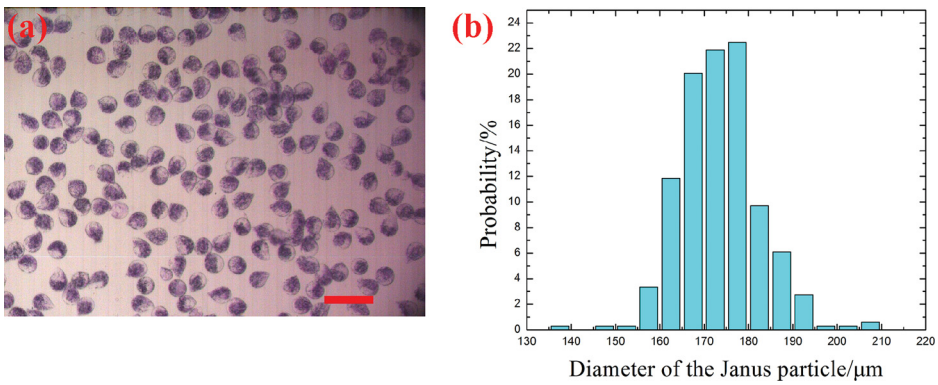

FIG. 3. (a) Optical microscope image (the scale bar is 500 μm) and (b) size distribution of Janus particles fabricated using our approach. The flow rate of the fluid is 5 ml/h and the electric field strength is 4.255×10^5 V/m.

particles is about 4%, as shown in Figure 3. A further increase in electric field strength results in oscillation of the tapered tip, leading to higher polydispersity in the droplet size.

Apart from the strength of electric field, the size of the droplets also depends significantly on the flow rate of the dispersed liquid.[20] We fabricate particles by electrospray at three different flow rates while keeping the electric field strength constant (Figures 4(a)–4(c)). The size of particles increases with increasing flow rate, as demonstrated in Figure 4(d).

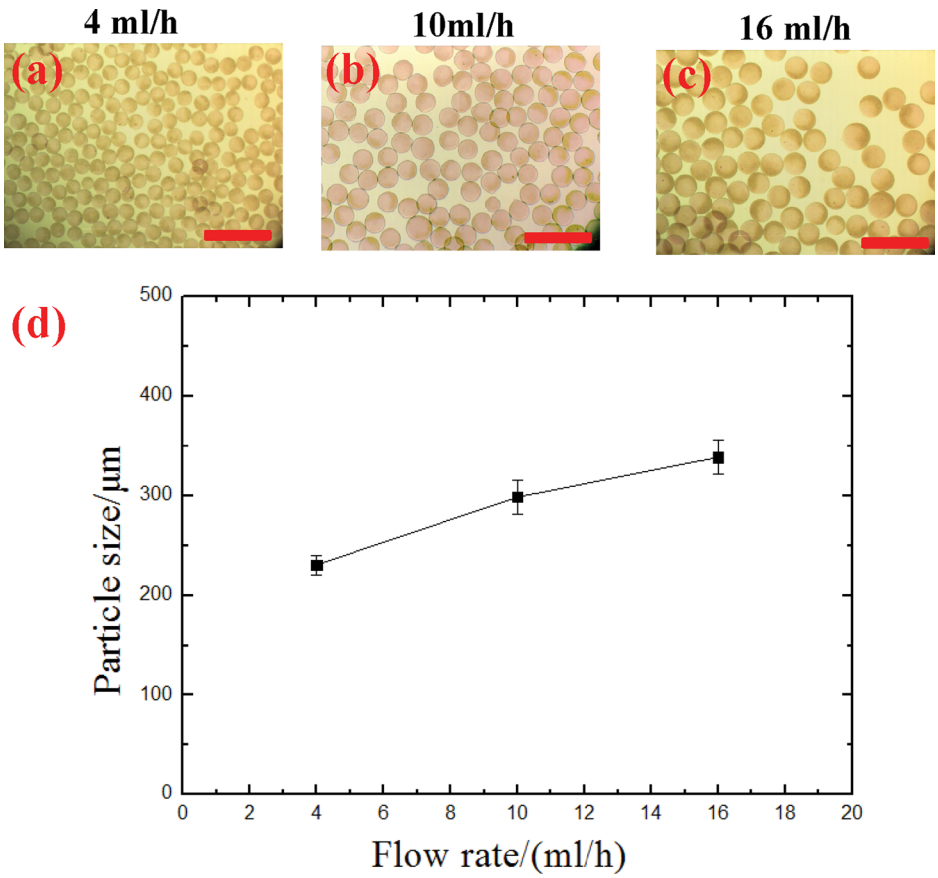

FIG. 4. Optical microscope images of Janus particles formed by electrospray with the fluid flow rate of (a) 4 ml/h, (b) 10 ml/h, and (c) 16 ml/h, respectively. (d) Effect of the fluid flow rate on the particle size. The electric field strength of these three cases is 3.17×10^5 V/m. The scale bar is 1 mm.

B. Particles with multi-compartment morphology

By controlling the electric field strength and the flow rate, we fabricate uniform particles using our combined approach of microfluidic and electrospray. Due to the low Reynolds number of the flow (typically less than 1), achieved by keeping the inner nozzle diameter to a few hundred microns, the mixing of the two streams is mainly caused by diffusion. As a result, the different dispersed fluids remain separated, without significant mixing and thus the multi-compartment morphology of the particles can be formed.[21] Indeed, the Janus character is not obvious as the size of the particles is reduced, due to mixing of the dye molecules that we use to track the interface (Figure 3(f)). When the droplet size decreases, the distance over which the dye molecules have diffused within a given time becomes comparable with the overall droplet size; as a result, the Janus character of the droplets is less distinguishable. However, complete mixing of the encapsulated cells due to diffusion is prevented as cells have a significantly larger size and thus a lower diffusion coefficient than the dye molecules. Moreover, for cell co-culture studies, the hydrogel particles need to be large enough for encapsulation of multiple cells, those particles with a diameter of at least several hundred microns will normally allow the distinct Janus character to develop. To demonstrate the potential of the approach for fabricating multi-compartment particles, we encapsulate different fluorescence dye molecules in the different compartments of the particles. This ensures that the multi-compartment structure can be identified by the different fluorescent colors (Figure 5). In this manner, we fabricate uniform Janus particles, with one side labeled by a red fluorescence color and another side highlighted by a green fluorescence color, as shown by Figure 5(a). Moreover, the relative volume fraction of each compartment in the particles can be tuned by changing the ratio of the flow rates of the two entering dispersed phases. By controlling the flow rate of the two dispersed phases, we fabricate Janus particles with two different volume ratios of 1:1 and 2:1, as shown in Figures 5(a) and 5(b), respectively. Particles with a larger number of compartments can be achieved by simply increasing the number of the input nozzles each containing different dispersed phases. We demonstrate this by preparing particles with red, green, and dark compartments, as shown in Figure 5(c). The impact of the sprayed droplets with the collecting solution often deforms their shapes; due to the fast crosslinking and the slow relaxation back to a spherical shape, some crosslinked alginate particles adopt a non-spherical tear-drop shape with tails.

C. Cell encapsulation and cell viability

Due to their similarity in structure with the extracellular matrix of cells, the alginate hydrogel particles provide promising micro-environments for encapsulation of cells.[22,23] The semi-permeable structure of the hydrogel allows the transport of the small molecules such as the

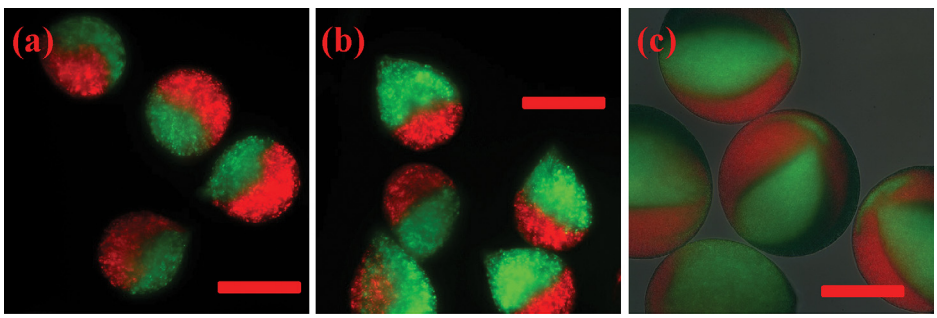

FIG. 5. Fluorescence microscope images of multi-compartment particles. Two kinds of Janus particles are presented: the volume ratios of the two sides are (a)1:1, (b) 2:1. (c) Microscope image of three-compartment particles. Conditions of fabrication for each image are as follows: Figure (a), flow rates are 2 ml/h in each side; applied electric field strength is 4.5×10^5 V/m; Figure (b), flow rates of the green and red precursor solutions are 4 ml/h and 2 ml/h respectively. The applied electric field strength is 4.5×10^5 V/m; Figure (c), flow rate of the precursor phases is 5 ml/h in each side while the applied electric field strength is 5×10^5 V/m. The scale bar is 200 μm.

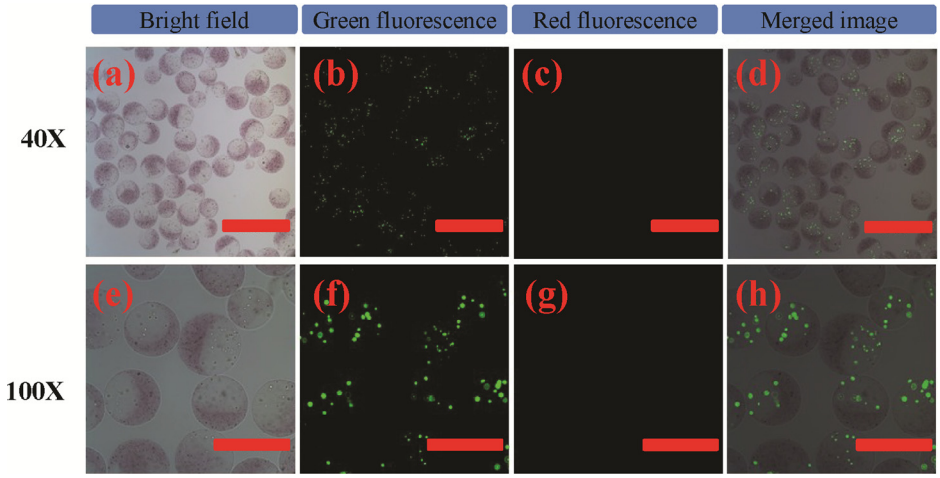

FIG. 6. Optical microscope images of Janus particles with magnifications of (a) 40 times, and (e) 100 times. (b), (c), (f), (g) Fluorescence microscope image of the Janus particles with stained cells encapsulated. Live cells are stained with a green fluorescent dye (calcein-AM), as shown in (b) and (f), while dead cells are stained with a red fluorescent dye (ethidium homodimer-1), as shown in (c) and (g); (d) and (h) are overlays of images captured by optical microscope and fluorescence microscope. The scale bar for the images with the magnification of 40 times is 1 mm while that for the images with the magnification of 100 times is 0.5 mm.

nutrients and biological factors while large molecules and particles, such as biological cells, remain immobilized. For the particles to be used in biological studies, the cells have to be viable inside them. To confirm that the cells are not harmed by the high voltage, we check the viability of the cells using a live/dead assay. Under the fluorescence microscope, living cells will show a green fluorescent color with the intracellular esterase indicated by the calcein-AM, while the dead cells will show a red fluorescence with the damaged membrane indicated by ethidium homodimer-1. Using the approach of microfluidic electrospray, Janus particles with 3T3 fibroblast cells encapsulated on one side and dye molecules encapsulated on the other side were fabricated, as shown in Figures 6(a) and 6(e). The number of cells per particle could be manipulated by varying the density of the cells in the suspension as well as the size of the bead. In our experiment, each particle contains 10 ± 2 cells on average. The Janus particles are then examined under the fluorescence microscope for confirmation of the viability of the cells. Almost all cells inside the Janus particles are alive, as shown by the green fluorescence (Figures 6(b) and 6(f)) and the absence of red fluorescence (Figures 6(c) and 6(g)). This indicates the high viability of the cells inside the multi-compartment particles and hence confirms that the cells have not been harmed by the high voltage. This agrees with results from a previous study suggesting that the high intensity of electric field does not cause noticeable harm to the cells.[24] During the fabrication process, the electric current was extremely low (less than 10^{-5} A) due to the low conductivity of air; this may explain why the cells are not harmed.

IV. CONCLUSION

In summary, we introduce a robust and reliable approach to fabricate monodisperse multi-compartment particles by combining the techniques of microfluidics and electrospray. These particles with cross-linked alginate chains as the matrix material have distinct compartments. By encapsulating different types of cells or cell factors in the different compartments, these multi-compartment particles can be used for cell co-culture studies. We also demonstrate that the cells encapsulated are not harmed during the fabrication process. Our approach therefore represents a simple technique for fabricating a cytocompatible micro-environment for cells. This platform has great potential for studying the cell-cell interactions as well as interactions of cells with extracellular factors.

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