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Designing compartmentalized hydrogel microparticles for cell encapsulation and scalable 3D cell culture

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Abstract

We describe here designs of compartmentalized hydrogel microparticles with tunable extracellular matrix (ECM) support for cell encapsulation and scalable 3D cell culture. The microparticles, rapidly formed by a one-step, multi-fluidic electrostatic spraying technique (>10,000/min), have a uniform spherical shape, a nearly monodisperse size distribution and controlled compartmentalization. They not only have a high surface area for mass transfer but also offer defined space and essential ECM support for various scalable and efficient 3D cell culture, co-culture and microtissue production applications.

Introduciton

Hydrogel microparticles have been used extensively for cell encapsulation, culture and transplantation.[1-6] The microparticles protect the cells from the environment, or the immune system when transplanted, while simultaneously allowing facile mass transfer necessary for the cell survival and function. They have therefore found tremendous applications in tissue engineering[7, 8] and cell therapy.[1, 6, 9] However, in many cases, the microparticles have no internal structure and the cells are encapsulated randomly in whatever material that forms the microparticles, most commonly alginate.[5, 10] It has now been increasingly recognized that controlling the structure and composition of these microparticles will significantly expand their applications. For example, alginate-based core/shell microparticles were made to improve the immunoprotection[11]; liquid-core microparticles were reported for confined 3D cell culture[12, 13]; and janus microparticles were used for co-encapsulation.[14, 15] While they had enhanced properties and functions, these structured microparticles had no natural extracellular matrix (ECM) support. In native states, cells are supported architecturally by ECM, and surrounded by other cell types; many cells require specific microenvironment to perform physiologically relevant functions.[16-18] For example, collagen particles have been shown to induce osteogenic differentiation of the encapsulated mesenchymal stem cells.[19] Thus, it is highly desirable to control the cellular environment within the microparticle platform.[20,21]

Here we report complex hydrogel microparticles with controlled ECM internal compartments for efficient and scalable 3D cell culture. Compared with conventional 3D culture methods where the cells are embedded in bulk ECM hydrogels (e.g. collagen), the microparticles have a larger surface-to-volume ratio for mass transfer and can be potentially cultured in suspension in stirred bioreactors for scale-up. We made the microparticles and encapsulated the cells by a one-step electrospraying-based, multi-fluidic cell micropackaging technique. The method allowed a high rate production (> 10,000/min) of compartmentalized

hydrogel microparticles with a uniform spherical shape and nearly monodisperse size distribution. The process is relatively simple and does not involve surfactants,[22] oils[21-23] or acids[22, 23] that are typically used in microfluidic flow focusing approaches. We demonstrated the versatility of these microparticles as an efficient and scalable 3D tissue culture platform through four different proof-of-concept examples. First, using an emerging model system of small intestinal organoids, we demonstrated the successful culture of therapeutically important cells within their preferred microenvironment in a microparticle format. Second, by taking advantage of the confinement effect of the microparticles, we showed scalable and robust productions of size-controlled multicellular tumor microtissues. Third, we demonstrated the utilization of the microparticles for studying the cell-cell interactions such as cell segregations under 3D confined space and the supporting role of stromal cells in maintaining *in vitro* culture of hepatocytes. Lastly, we showed the possibility of combining these uniform microparticles with addressable micro-well systems for potential reconfigurable paracrine cell co-culture applications.

Results and Discussion

We made the compartmentalized hydrogel microparticles by adopting a multi-fluidic electrostatic spraying technique. (See Figure S1 for schematics and details.) A similar technique has been previously used to prepare structured solid polymer particles.[24, 25] However, here we used it to produce spherical, nearly monodisperse hydrogel microparticles including a novel triple-layer concentric configuration for cell encapsulation. Figure 1 shows three different microparticle designs (double-layer, side-by-side and triple-layer), all from Ca^{2+}/Ba^{2+} - crosslinked alginate hydrogel. The alginate in different compartments was labeled with a different fluorescent color for visualization purpose. Using model cells (MDA-MB-231 expressing GFP, normal human lung fibroblasts (NHLFs) with RFP and MCF-10A stained with Hoechst), we demonstrated that different types of cells could be encapsulated in distinct

compartments within individual particles. With a uniform spherical shape, a nearly monodiperse size distribution and a high production rate, these hydrogel particles are in contrast with those made previously by the high flow rate jetting[12] or centrifuge-based[14] approaches that produced non-uniform microparticles or the sequential deposition method that was difficult to scale up.[26] Moreover, the complex hydrogel microparticles described here are also unlike the previously reported structured particles that were made of solid polymers and might not be suitable for cell encapsulation applications.

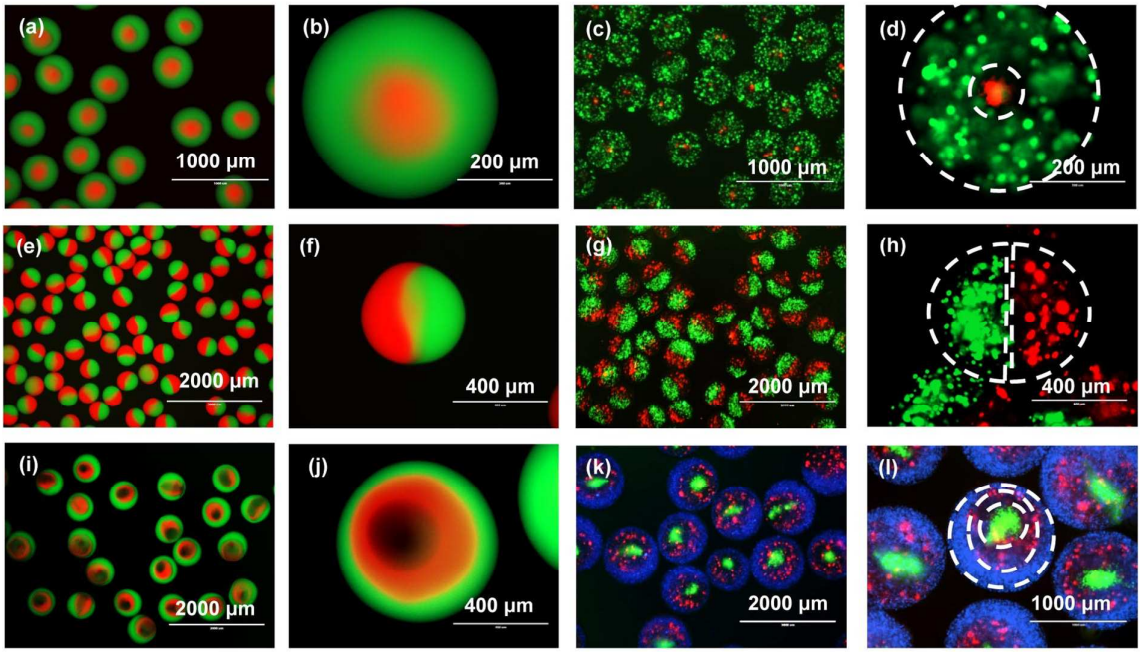

Figure 1. Hydrogel microparticle designs and their applications for cell encapsulation. (a-b) Double-layer hydrogel microparticles made of fluorescently labeled alginate (Red: alginate labeled with Alexa Fluor® 594 dye; Green: alginate labeled with Alexa Fluor® 488 dye). (c-d) Double-layer alginate microparticles encapsulating different types of cells (Green cells: MDA-MB-231 expressing GFP; Red cells: normal human lung fibroblasts expressing RFP). (e-f) Side-by-side alginate hydrogel microparticles. (g-h) Cell encapsulation using side-by-side microparticles. (i-j) Triple-layer hydrogel microparticles. (The inner most layer in i and j was unlabeled alginate.) (k, l) Cell encapsulation using triple-layer particles. (The blue cells in k and l were MCF-10A stained with Hoechst).

To incorporate ECM into the hydrogel particles, we simply replaced one or more inner alginate fluids with ECM hydrogel precursor solutions while keeping the outer alginate fluid the same. (See Figure 2a, Figure S2a, b and Figure S3a, b for schematics.) The microparticles were formed by an ionic crosslinking of alginate outer layer followed by a thermal (37 ^{o}C)

crosslinking of ECM inner layers. ECM hydrogels such as Matrigel™ provide physiologic growth environments and it is often desired to process them into robust microparticles for cell encapsulation applications. By using the multi-fluidic electrostatic cell micropackaging, we can encapsulate and culture various types of cells in different ECM compartments inside microparticles. (See Table S1 for a summary.) The particles not only have large surface area for mass transfer but can also be suspended in stirred bioreactors for large scale cell culture and expansion. Figure 2b-d shows the hierarchical structures of double-layer microparticles with a cell-containing ECM inner layer (collagen). (Also see Figure S2c-e for particles with an alginate outer layer and two different ECM cores, and Figure S3c-e for triple-layer concentric particles with an alginate outer layer and two different ECM inner layers.) In Figure 2d, the type I collagen fibers surrounding the cells in the microparticle were observed by the second-harmonic generation microscopy.[27] To demonstrate the unique applications of the ECM-containing microparticles, we first cultured small intestinal organoids. The small intestinal organoids have recently emerged as an important platform for both basic stem cell research and potential development of regenerative therapies.[28] It has been shown that the small intestinal crypts contain Lgr5+ intestine stem cells and when isolated have the capability to grow into crypt-villus structures or organoids that recapitulate the native intestinal epithelial functions and signatures.[29, 30] However, these crypts typically require specific environment to grow, and current culture methods such as embedding in bulk Matrigel[31-33] or Collagen[34] are well-established for crypts culturing (Figure S4). However, the double-layer hydrogel microparticles represent a new and potentially scalable platform to culture the crypts. The inner, Matrigel layer provides the neccessary microenvironment, while the outer, alginate layer forms robust microparticles that have increased surface-to-volume ratio and reduced diffusion distance and again can be produced continuously more than 10,000 per minute (see Experimental Section for details). Note that in simple alginate microparticles the crypts could not survive (Figure 2e), while in the double-layer

microparticles they grew into structured organoids with an enriched GFP-labeled Lgr5+ stem cell population as shown in Figure 2f.

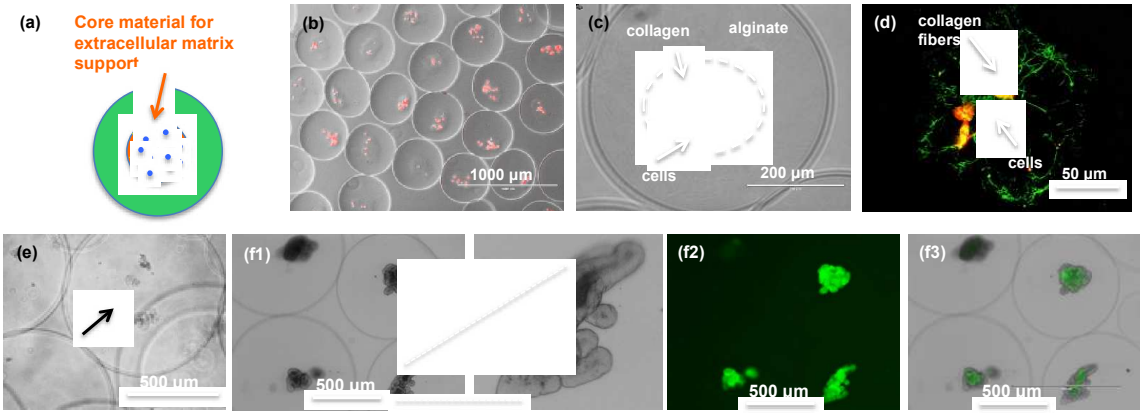

Figure 2. Hydrogel microparticles with ECM support for scalable 3D cell culture. (a) A schematic of an ECM-supported microparticle. (b) MDA-MB-231 cells with tomato red expression encapsulated within collagen matrix inner layer in the alginate microparticles. (c) A maginified view of a single microparticle. (d) Cells in the collagen fibers (green) in the inner layer of a microparticle as imaged by second harmonic generation microscopy. (e) Mouse small intestinal crypts encapsulated in alginate-alone microparticles failed to survive after 2 days of culture. (f) The crypts encapsulated in Matrigel inner layer of the microparticles grew into structured organoids in a week with an enriched GFP-labeled Lgr5+ stem cell population (f1: bright field; f2: GFP; f3: merged).

Next, we demonstrated the ECM-containing microparticles represented an excellent platform to generate size-controlled tumor microtissues. Tumor microtissues have many applications in drug screening, cancer modeling and therapeutic development.[35, 36] Several approaches have been successfully used to make the tumor microtissues such as the hanging-drop method.[37] However, this method requires manual seeding droplets and is difficult to scale up. In contrast, the microparticles can be continuously produced and suspended in stirred bioreactors for large-scale microtissue production. Previously, liquid-core microparticles made via the high flow rate jetting approach were proposed as a new platform to produce tumor microtissues and study the effect of confinement on their invasiveness.[12] However, more than half of the produced particles had poor shapes, making the control of microtissue size difficult.[12] Here we demonstrate the scalable production of microtissues from two different breast epithelia cells, nonmalignant MCF-10A (Figure 3a) and invasive MDA-

MB-231 (Figure 3b) using our alginate/ECM double-layer microparticles. Interestingly, the cells grew in the Matrigel inner layer and stopped growing (or grew with a much slower rate) after they filled the inner layer and reached the alginate/Matrigel interface (Figure 3c). This is consistent with the observation that these cells did not proliferate in microparticles composed of alginate alone. (Figure S5) The confined growth in the microparticles enabled us to control the microtissue size, from 95 μm to 725 μm, by simply changing the size of the Matrigel inner layer (Figure 3d and Figure S6). Previously, large size (up to 600 μm) tumor microtissues were considered to better mimic the primary tumor before vascularization and have been difficult to produce using conventional microfabricated cell culture platforms. [38] The 3D confinement of microparticles also made structurally defined microtissues than other unconfined systems such as bulk hydrogel or microwells for the E-cadherin-lacking MDA-MB-231 cells[39, 40] (Figure S7). After formation in the particles, the microtissues were easily recovered by dissolving the alginate outer layer using an ethylene diamine tetraacetic acid (EDTA) solution (Figure 3a4 and 3b4). As expected, the cells in the microtissues exhibited a size-dependent, heterogeneous viability. While the small size microtissues had mostly live cells, the large ones had necrotic centers (Figure S8), typical of non-vascularized primary tumors.[41, 42] During the course of microtissue growth, the hydrogel microparticles were stable with limited swelling (Figure S9), consistent with previous report that the addition of Ba^{2+} into the Ca^{2+} crosslinking bath made the alginate microparticles stronger. [43] It is noted that for some other types of cells such as Ins-1 cells (Figure S10), the microtissues may continue to grow after they fill the ECM core and eventually break the microparticles.

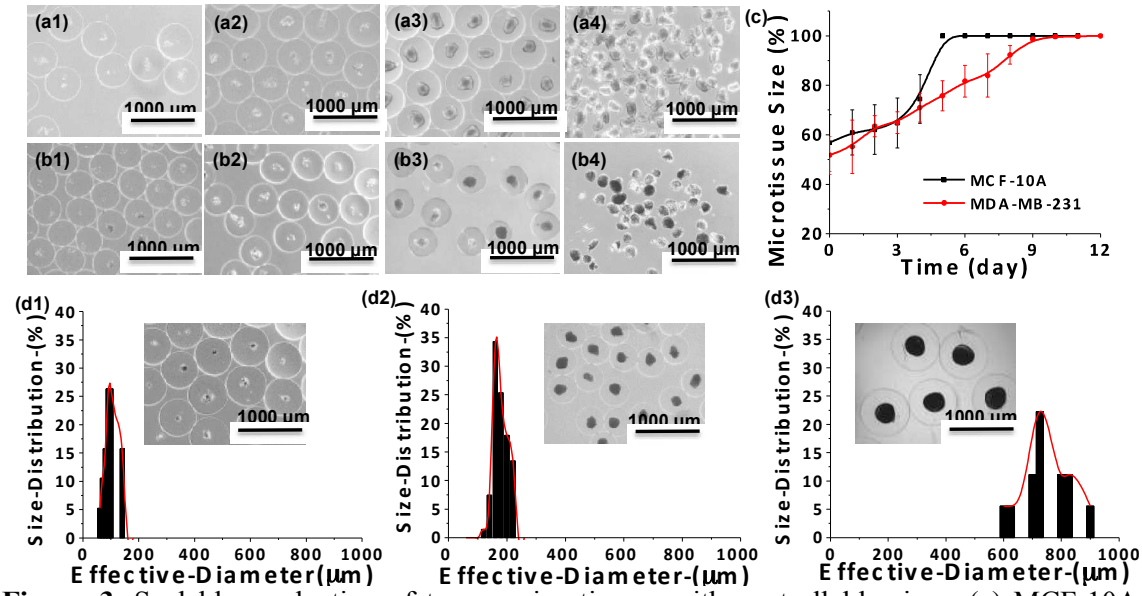

Figure 3. Scalable production of tumor microtissues with controllable sizes. (a) MCF-10A cells within Matrigel matrix in the microparticles, observed on day 0 (a1), day 3 (a2) and day 8 (a3). The microtissues (a4) were recovered by dissolving the microparticles using EDTA. (b) MDA-MB-231 cells on day 0 (b1), day 2 (b2), day 19 (b3) and microtissues recovered (b4). (c) The growing curve of these two types of cells. The microtissue size is defined as the mean of the longest and shortest dimensions of the microtissue. The average size is typically taken from about 50 microtissues. (d) The control of the average microtissue sizes: 95 μm (d1), 160 μm (d2) and 725 μm (d3).

Another application of these microparticles is for studying the cell-cell interactions in confined 3D co-cultures. Two or more different types of cells at different number ratios can be encapsulated together in the ECM inner layer of individual particles. As an example, we encapsulated the MDA-MB-231 cells and the MCF-10A cells at a ratio of 1:1 in the alginate/Matrigel microparticles. Remarkably, a random mixture of cells evolved over time into a well-defined, core-shell structure with the MDA-MB-231 cells (expressing dTomato fluorescence) enclosing the MCF-10A cells. (Figure 4a) The segregation and formation of boundaries between different cell populations are common and essential in tissue development and morphogenesis. [39, 44, 45] Here the double-layer particles provide a robust platform to study the cell segregation under 3D confined space. One question was why the benign cells (MCF-10A) aggregated in the center, while the more invasive ones (MDA-MB-231) were segregated in the peripheral. According to the differential adhesion hypothesis

(DAH), the different degrees of surface adhesion induce spontaneous cell reorganization to minimize the interfacial free energy. [46] It was also shown that two types of cells with differential E-cadherin expression segregated spontaneously with lower E-cadherin expression level cells enveloping higher expression level ones.[47] In our case, the MDA-MB-231 cells lack the E-cadherin and the MCF-10A cells express higher levels of E-cadherin, leading to the unique segregation within the microparticles.

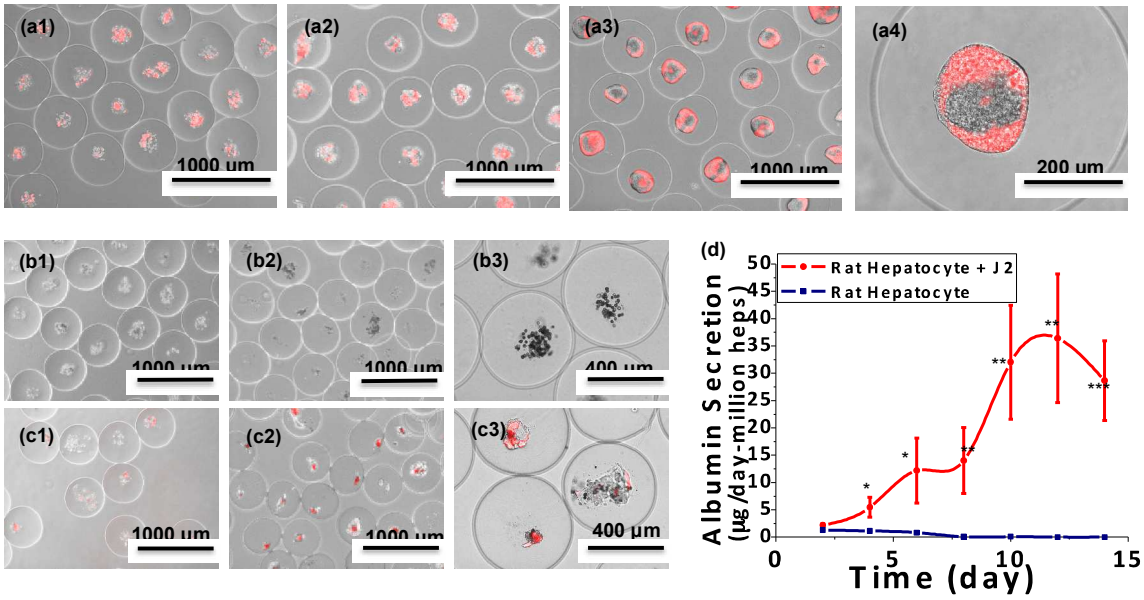

Figure 4. Cell-cell interactions in confined 3D cell co-cultures. (a) Interactions of breast epethelial cell lines, MDA-MB-231 (with red dTomato fluorescence) and MCF-10A cells. The cells over time segregated into core-shell structures: day 0 (a1), day 4 (a2) and day 7 (a3, a4). (b) Rat hepatocytes cultured alone in the Matrigel-supported microparticles: day 0 (b1); day 14 (b2, b3). (c) Rat hepatocytes co-cultured with stromal cells in the microparticles: day 0 (c1); day 14 (c2, c3). The stromal cells were mitotically inactivated, non-proliferating, mCherry-expressing (red) mouse 3T3-J2 fibroblasts. (d) The co-cultured hepatocytes had improved viability over time as measured by the albumin secretion (mean ± SE; n=6; ***p<0.01; **p<0.05; *p<0.1).

In addition to cell segregation, the cell-cell interaction also plays an essential role in cellular functions. Using the ECM-supported microparticles, we co-cultured hepatocytes and stromal cells and obtained drastically improved survival of hepatocytes. *In vitro* culturing of primary hepatocytes, particularly in a scalable fashion, has important applications in drug

screening and toxicity testing for pharmaceutical industries.[48, 49] However, the cell viability and hepatic functions decline rapidly *ex vivo*, under conventional culture conditions.[50, 51] Previous studies[52-54] have shown that a combination of ECM substrate and stromal cell support could enhance the hepatocyte survival and stabilize their functions. Although several techniques[55, 56] have been reported to realize this combination, most of them were essentially two-dimensional (2D) cultures. Our ECM-supported microparticles offer a 3D, miniaturized culture environment with a great potential of scalability and parallel culturing. We first encapsulated the rat hepatocytes alone in the alginate/Matrigel microparticles (Figure 4b1) and after 14 days of culture the cells appeared either dead or loosely dispersed in Matrigel (Figure 4b2, b3). In contrast, when we co-encapsulated the hepatocytes and the non-proliferating, mitotically inactivated mouse 3T3-J2 fibroblast stromal cells at a ratio of 3:1 (Figure 4c1), the cells were aggregated together and appeared mostly healthy (Figure 4c2, c3) after 14 days. The secretion of albumin measured by ELISA (enzyme-linked immunosorbent assay) confirmed the significant improvement in hepatocyte viability with the co-culturing, as shown in Figure 4d. The dead/live staining from a separate experiment also confirmed the improved survival of hepatocytes when co-encapsulated with stromal cells (Figure S11).

Lastly, to demonstrate the applications of the microparticles for potential screening and 3D paracrine (non-contact) cell co-cultures, we combined them with an individually addressable microwell system.[44, 57, 58] We seeded the microparticles (~470±30 μm) into PDMS microwells (500 μm depth and 500 μm diameter). Figure 5a shows a schematic and representative images of the fluorescently labeled alginate microparticles seeded in microwells. Microparticles with GFP-expressing HUVECs (human umbilical vein endothelial cells) encapsulated in the fibrin hydrogel were seeded in the microwells similarly (Figure 5b). HUVECs are widely used for vascular engineering[59, 60] and studies of angiogenesis and vascular biology.[59, 61, 62] They are typically cultured in ECM such as fibrin hydogels. Here we

culture the HUVECs in fibrin within arrayed microparticles, which may find applications for screening of angiomanipulatory drugs.[59, 63] (Note that the HUVECs could not survive in conventional, alginate alone microparticles – see Figure S12.) Furthermore, the "microparticles in microwells" configuration represents a new, potentially scalable platform for 3D paracrine cell co-culture [64, 65] where one type of cells are cultured in microparticles and the second type of cells are seeded in microwells. To provide a proof of concept, we first aggregated the RFP-expressing normal human lung fibroblast (NHLF) in the microwells and then seeded the microparticles with HUVECs, as shown in Figure 5c. One advantage of this sytem compared with previously published ones such as the InVERT molding[55] or suspending microparticles in bulk hydrogel[64] is that the microparticles and the microwells are detachable and switchable. Thus, this approach provides opportunities for reconfigurable cell co-cultures.

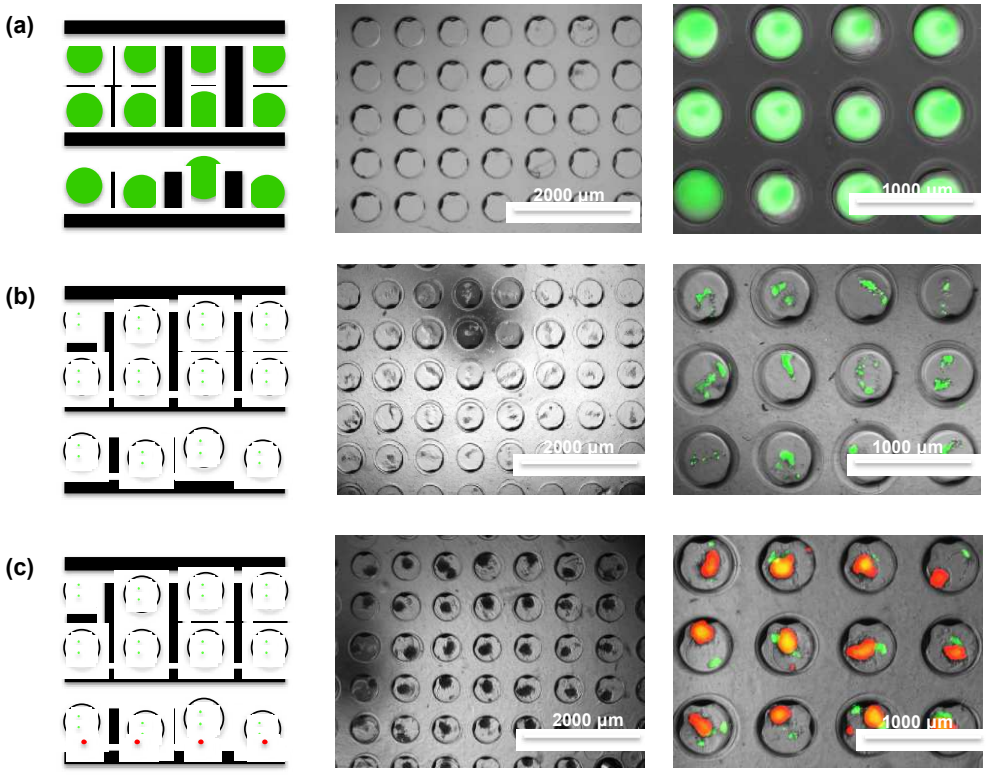

Figure 5. "Microparticles-in-microwells" culture systems. The schematics illustrate the configurations with both the top and side views. Both the bright field images and the merged images with fluorescent colors are shown. (a) Fluorescently labeled alginate microparticles seeded in microwells. (b) Alginate/fibrin double-layer microparticles encapsulating GFP-

expressing HUVECs seeded in the microwells. (c) HUVECs (green) -containing microparticles seeded in microwells that were pre-loaded with NHLF cells (red).

Conclusion

In conclusion, we report here compartmentalized hydrogel microparticles and their potential applications for efficient and scalable 3D cell culture. The microparticles were produced by a multi-fluidic electrostatic spraying technique with a nearly monondisperse size distribution at a rate of over 10,000/min. The uniform size, high production rate and possibility to incorporate ECM make these particles a versatile platform for various cell culture applications in a potentially scalable manner. We demonstrated the culturing of small intestinal organoids and production of size-controlled tumor microtissues using the microparticles. By co-encapsulating two different human breast epithelial cell lines, the invasive MDA-MB-231 and the benign MCF-10A, inside the microparticles, we observed a cell segregation under 3D confined space. We are performing more mechanistic studies and exploring the implications of this phenomenon in relation to malignancy[66-68] and the potential applications in cancer diagnostics. The use of these microparticles as a scalable platform for 3D co-culture was further demonstrated in a hepatocyte/stroma co-culture system where as expected the hepatocyte viability was significantly improved with stromal cells. Finally, we constructed a reconfigurable, "microparticles-in-microwells'' paracrine co-culture system. The microparticles provide the physiologically-relavent 3D extracellular microenvironment with facile mass transfer, while the microwells offer the convenient, chip format adaptable to parallel, *in situ* imaging and screening applications.[52] Taken together, the data presented in this paper show the great potential of custom designed hydrogel microparticles for efficient and scalable 3D cell cultures.

Experimental Section

More details are given in the Electronic Supporting Information

Production of hydrogel microparticles

Multiple fluids were pumped into a high electrical field through a multi-channel nozzle. Depending on the configuration of the microparticles, different nozzles were used (See Figure S1 for details). The outer fluid was typically 0.9% (w/v) sterile alginate (UPLVG FMC Biopolymers) dissolved in 0.8% (w/v) sodium chloride. The viscosity of the alginate solution was 94 mPa*S at 4 ^{o}C. The inner fluids were the cells mixed with appropriate medium and ECM components. The viscosity of a typical Matrigel cell-suspension was 1.72 mPa*S at 4 ^{o}C. As the strength of electrical field increases (typically to ~ 6 kV), compound droplets were formed and collected in the crosslinking bath with 100 mM calcium chloride and 5 mM barium chloride, located 1.8 cm beneath the nozzle. All the reagents and the nozzles/tubes were pre-cooled to 4 °C to avoid gelling of the ECM components during the process. The microparticles were spherical and uniform (see Figure S13 for one example) and their diameter was controlled by tuning the total flow rates and voltage. The size of inner ECM compartments was controlled by changing the relative flow rates of the outer and inner fluids (Figure S6). The typical flow rates for the outer fluid was 0.3-0.45 ml/min and that of inner fluids were from 0.005 to 0.025 ml/min. Given an average microparticle diameter of 420 μm, the production rate was approximately 13,000 per minute.

Formation of ECM hydrogel within the microparticles

The ECM we used in the microparticles included Matrigel^{TM} (BD Biosciences), type I collagen and fibrin. Matrigel^{TM}, a mixture of laminin, collagen type IV, and enactin with many different growth factors[69, 70] was diluted with appropriate growth medium to a 16.7% (v/v) solution at 4°C. After encapsulation, the gelation of Matrigel^{TM} occurred at 37°C in the

incubator. Type I collagen was extracted from rat tail tendon in 0.1% (v/v) acetic acid at 4 °C for 3 days. After centrifugation, the supernatant was removed and frozen at -20 °C. It was lyophilized and then the pellets were reconstituted in 0.1% acetic acid at 4 °C for 3 days at a desired concentration (5 mg/ml). Before use, it was diluted with growth medium and neutralized by 1N sodium hydroxide at 4°C. The final concentration of collagen for encapsulation was about 0.45 mg/ml. The collagen gel/fibers were formed after the microparticles were incubated in the growth medium at 37°C for 30 minutes. Finally, the fibrinogen (Sigma) were diluted with growth medium to a 2.5 mg/ml solution. The microparticles were put in growth medium with 3 unit/ml thrombin (Sigma). The fibrin hydrogel in the microparticles were subsequently formed at 37°C in the incubator.

Cell culture

Malignant MDA-MB-231 breast adenocarcinoma cells and normal human lung fibroblasts (NHLFs) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies), which is supplemented with 10% fetal bovine serum (Life Technologies) and 1% penicillin-streptomycin (Life Technologies). MCF-10A mammary epithelial cells were maintained in DMEM:F12 supplemented with 5% horse serum, 0.5 μg/ml hEGF, 10 μg/ml insulin (Sigma-Aldrich), 100 ng/ml cholera toxin, and 100 units/ml penicillin and 100 μg/ml streptomycin. MDA-MB-231 and MCF-10A were stably transduced with lentivirus encoding genes for dTomato and EGFP fluorescent proteins, respectively. Each transfected cell line was cultured in its original medium. INS-1 832/13 cells were cultured in RPMI supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10% fetal bovine serum, 10 mM HEPES, 1% penicillin-strepomycin, 250 ng-ml-1 amphotericin B, and 50 μM β-mercaptoethanol. Human umbilical vein endothelial cells were cultured in EGMTM-2 Bulletkit™ (Lonza). The small intestinal crypts were isolated from mice based on a method as previously described [31] and cultured in a previously published medium.[33] Primary rat hepatocytes were isolated and

purified by a modified procedure of Seglen[71] and maintained in DMEM medium with 10% fetal bovine serum, supplemented with insulin, hydrocortisone, and antibiotics. All cells were cultured at 37℃ and 5% CO_2 in a humidified incubator. The cells (~20-30 million cells/ml ECM) were encapsulated in a process similar to the microparticle production as described above. After encapsulation, the microparticles with cells were cultured in appropriate growth medium.

Electronic Supporting Information

Supporting Information is available online.

Acknowledgements

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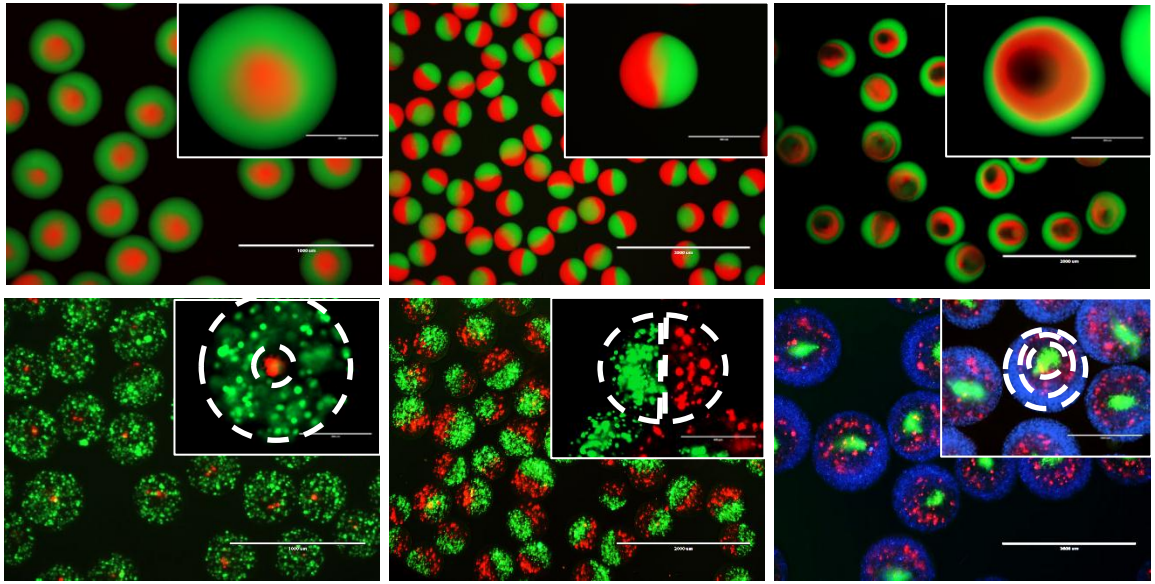

Compartmentalized hydrogel microparticles with high production rate, uniform size and shape, and tunable ECM support were developed for various scalable 3D cell culture applications.