Fabrication of Hydrogel Particles of Defined Shapes Using Superhydrophobic-Hydrophilic Micropatterns

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Hydrogels are hydrated cross-linked polymers resembling natural extracellular matrix that provide soft three-dimensional (3D) support for cellular growth and tissue formation.[1] Due to their unique properties such as high porosity, permeability for gases, nutrients, and metabolites, as well as their compatibility with physiological conditions, hydrogels have been extensively studied as material support for immobilizing cells, cultivating cells in 3D,[2] biomedical applications, drug delivery, and tissue engineering.[3]

Two distinct approaches namely “top-down” or “bottom-up” have recently emerged in hydrogel engineering. Top-down approaches control the microscale features (i.e., size and shape) of relatively large pieces of bulk hydrogels,[4] whereas bottom-up approaches aim to generate larger tissue constructs via the assembly of smaller building blocks (usually cell-laden hydrogels) which mimic the tissue structure consisting of repeating functional units.[5]

The limitations of bulk hydrogels are that they usually lack the hierarchical architecture of tissues and suffer from the slow diffusion of nutrients and other biological signaling molecules (e.g., growth factors) from the surrounding medium into the entrapped cells, leading to higher toxicity and incompatibility with long-term cell cultivation. These drawbacks undermine the concept of employing hydrogels for 3D cell cultivation.[6,7]

Several attempts have been proposed to solve this problem.[8–12] The Doyle group developed methods to generate hydrogel microparticles with specific shapes using continuous flow or stop-flow lithography.[13] A droplet-based microfluidic system was proposed to construct alginate gel beads encapsulating cells.[14] Cell-encapsulating hydrogel particles have recently been used in several fields such as 3D cell culture and in vitro microphysiological models.[15,16]

However, there are still too few methods allowing for the fabrication of hydrogel particles with different geometries and dimensions compatible with the cell encapsulation.[17] New approaches combining the encapsulation of cells into hydrogel structures with complex geometries and long-term cell viability are also required.

We recently demonstrated a versatile platform for creating thousands of isolated microdroplets of specific geometry and volume, based on the use of superhydrophobic (SH) surfaces patterned with wettable superhydrophilic (SL) domains.[18–22] The extreme wettability contrast of the SH–SL patterns allows the spontaneous separation of an aqueous solution into high-density arrays of microdroplets using the effect of discontinuous dewetting. This rapid and facile droplet formation does not require multiple pipetting or a liquid handling device. The handling of small volumes of droplets requires fewer reagents than with conventional microplates.

Microarray platforms can be used to study cellular behavior by creating distinct combinations of chemical/biochemical microenvironments.[23,24] Micropatterning and microfluidic systems have been used to create hydrogels with spatially controlled organization as well as numerous cell-material or cell–cell combinations.[25,26] Hydrogel microarrays have been used for various applications, including disease diagnosis, prognosis, biochemical analysis, and high-throughput multiplexed assays.[27–30] Strategies avoiding fixation of various hydrogels in the 2D format have employed shape-coded hydrogel particles as a suspension microarray format for multiplexed bioassays.[31,32]

Here, we describe a method for the rapid fabrication of alginate hydrogel particles of defined sizes and shapes using the effect of discontinuous dewetting on an SH–SL microarray combined with the sandwiching method to achieve the simultaneous gelation of the prehydrogel droplets. We applied this method in three demonstrations: (1) preparing an array of hydrogel particles and freestanding hydrogel particles with distinct geometries and sizes defined by the photomask features; (2) examining the viability of cells encapsulated into freestanding hydrogel particles, and (3) constructing magnetic responsive hydrogel particles for modular tissue engineering and shape-coded freestanding hydrogels of distinct cell types.
To produce the SH–SL array, a nanoporous poly(2-hydroxyethyl methacrylate-co-ethylene dimethacrylate) layer was formed on a microscope glass slide followed by modifying the polymer surface with alkyne groups via esterification. Afterward, an SH pattern with specific geometry was formed by functionalizing the surface with 1H,1H,2H,2H-perfluorodecanethiol using the thiol-yne photo-click reaction and by applying a corresponding quartz photomask. The remaining alkyne groups were reacted with 2-mercaptoethanol under UV light to form the desired pattern of superhydrophilic areas separated by superhydrophobic borders. The porous SH regions possessed advancing ($\theta_{\text{adv}}$), static ($\theta_{\text{st}}$), and receding ($\theta_{\text{rec}}$) water contact angles (WCAs) of 173°, 170°, and 164°, respectively, while the SL areas showed WCAs <10°.

The extreme difference in wettability between the SL spots and SH background creates a means of forming arrays of microdroplets using the effect of discontinuous dewetting (Figure 1, step 1) (Movies S1 and S2, Supporting Information). The size and volume of the droplets depend on the geometry and surface area of the hydrophilic regions and is controllable from 700 pL up to 3 μL. This allows for the dispensing of aqueous solutions into thousands of droplets without the need for manual pipetting or robotic devices. As soon as the nanoporous SL areas become wet, the porous polymer becomes transparent due to reduced light scattering caused by matched refractive indexes, thereby making SL spots visible and enabling light and fluorescent microscopy analysis. In this project, we prepared arrays of droplets of different shapes and sizes (Figure 2; Figure S1, S2, Supporting Information).

The workflow of the high-throughput fabrication of hydrogel particles is illustrated in Figure 1. In the first step, two separated arrays of droplets were formed. Slide 1 was prepared using a nano-porous poly(2-hydroxyethyl methacrylate-co-ethylene dimethacrylate) layer, followed by modifying the polymer surface with alkyne groups via esterification. Afterward, a SH pattern with specific geometry was formed by functionalizing the surface with 1H,1H,2H,2H-perfluorodecanethiol using the thiol-yne photo-click reaction and by applying a corresponding quartz photomask. The remaining alkyne groups were reacted with 2-mercaptoethanol under UV light to create the desired pattern of superhydrophilic areas separated by superhydrophobic borders. The porous SH regions possessed advancing ($\theta_{\text{adv}}$), static ($\theta_{\text{st}}$), and receding ($\theta_{\text{rec}}$) water contact angles (WCAs) of 173°, 170°, and 164°, respectively, while the SL areas showed WCAs <10°.

Figure 1. Schematic representation of the droplet microarray platform and workflow for the high-throughput fabrication of hydrogel particles via the sandwiching method. Step 1: Formation of an array of droplets of a prehydrogel solution on a superhydrophobic–superhydrophilic array. Step 2: Crosslinking of alginate droplets by performing parallel addition of CaCl₂ solutions into the individual droplets via the sandwiching method. By changing the position of the slide 1 (bottom vs top) containing CaCl₂ droplets, it is possible to form either an array of fixed hydrogel particles (Step 2a) or detach hydrogel particles to form freefloating hydrogel particles (Step 2b). Scale bar: 2 mm.
CaCl\textsubscript{2} solution (1 mol L\textsuperscript{-1}) as a cross-linker. The second slide (Slide 2) contained cells suspended in 2 mg mL\textsuperscript{-1} of alginate solution. Afterward, the two slides were aligned to come briefly into contact (Slide 1 – bottom, Slide 2 – top) to mix and form separated hydrogel droplets (Figure 1). The droplets’ gelation occurred within seconds (~10 s) after the addition of CaCl\textsubscript{2} solution. Then, the two slides were separated and an array of adhered hydrogel particles formed on the slide, which was located at the bottom during sandwiching (Figure 1, step 2a). In this case, our hypothesis is that CaCl\textsubscript{2} trapped in the porous polymer continues to diffuse into the droplets after the sandwiching step, leading to the formation of a stronger hydrogel layer at the surface, which anchors hydrogel to the polymer substrate even when the slide is immersed into water (Movie S3, Supporting Information). The culturing of hydrogels-on-chip for 7 d fully immersed in medium did not result in detachment. By changing the position of the two slides (Slide 1 – top, Slide 2 – bottom) an array of hydrogel particles formed again (Figure 1, step 2b). In this case, hydrogel particles could be easily detached from the Droplet Microarray (DMA) slide by immersing it in culture medium or buffer, thereby forming freestanding hydrogel particles containing cells (Movie S4, Supporting Information). We hypothesize that the reason for the weaker adhesion of the hydrogel particles in this case is the fast consumption of lower amount of CaCl\textsubscript{2} available for the cross-linking process in the droplets resulting in a weaker hydrogel layer at the bottom of droplets and their easy detachment.

To demonstrate the broad applicability of this method, we used the DMA platform to fabricate a variety of hydrogel particles with different geometries (Figure 2). Both freestanding and on-array hydrogel particles of different sizes and geometries could be prepared (Movies S5–S8, Supporting Information). The dimension and shape of the hydrogel particles can be controlled by the photomask design. Comparison of the fluorescence intensity of hydrogels formed on distinct SL spots, as well as the dimensions of the freestanding hydrogels proved the homogeneous size distribution of hydrogel particles of different shapes (Figure S2, Supporting Information).

Figure 2 demonstrates that the droplet array method is not perfectly suited for the formation of hydrogel particles with sharp corners. This is a result of the surface tension of water solutions that tries to round off the corners of liquid droplets at
the sharp edges of the hydrophilic–hydrophobic patterns. However, the shapes of hydrogel particles are easily recognizable and could be distinguished automatically by an image-recognition software. It is also evident from the images in Figure 2 that the geometry of edges of the pattern (straight lines for triangles and hexagons; round, or even concave in the case of hearts) is transferred to the hydrogel particles.

The shape of hydrogel particles can be applied in various ways and can also have an effect on cells or cell encapsulation. For example, the size of particles will determine the number of cells per particle. The shape of particles will be important for constructing macroassemblies with diverse architectures, interparticulate distances or particle-to-particle interactions. This method can be used as a toolbox of cell bearing hydrogel bricks of different shapes to enable diversity of multiparticle assemblies for modular tissue engineering.[29] In addition, shape of such building blocks can be used to encode information about the composition of the individual blocks without the need for separate fluorescent labeling (vide infra).

The mechanical properties of hydrogels are important for mimicking the in vivo cellular environment and for achieving higher cell viability during long-term cell culture. The stiffness of hydrogels depends on the concentration of CaCl2 used for gelation. Dynamic mechanical analysis (see the Supporting Information and Figure S1e,f [Supporting Information] for details) was used as a nondestructive technique to characterize the viscoelasticity of the hydrogels fabricated using different concentrations of CaCl2. To check the long-term stability of freestanding hydrogels, we assessed the mechanical and viscoelastic properties on hydrogels immersed in cell culture medium with or without the supplementation of 1% (v/v) of CaCl2 solution for 1, 3, 5, and 7 d (see Figure S3 for Supporting Information). Our results revealed that after immersion of cross-linked alginate hydrogels into cell culture medium the hydrogels' storage modulus decreased from 95 kPa (day 1) to ≈19 kPa on day 7 (Figure S3a, Supporting Information).[34] This could be explained by ionic exchange between calcium and monovalent ions (such as sodium ions). However, this tendency seemed to be reversed by adding calcium ions to the medium. The hydrogels' storage modulus increased with the duration of immersion in the calcium-supplemented medium, to ≈200 kPa, after 7 d of immersion (Figure S3b, Supporting Information). Moreover, in hydrogels immersed in calcium-containing medium, the loss-factor values did not vary significantly with time (Figure S3c,d, Supporting Information).

In the next step, we also fabricated freestanding hydrogel particles incorporating living cells (Figure 3a). We first demonstrated that alginite and CaCl2 exerted no cytotoxic effect on live human cervical tumor cell line expressing GFP (HeLa-GFP) (Figure S4, Supporting Information). Then, cells were seeded onto the SH–SL patterned surface containing round SL spots of 3 mm diameter by applying cell suspension containing 2 mg mL\(^{-1}\) of alginate to form an array of separated droplets. CaCl2 solution was spread on a separate DMA slide. Hydrogels were formed by sandwiching both slides using the sandwiching device (Figure 1). This led to the formation of an array of round, freestanding hydrogel particles of 3 mm diameter at the base. Each hydrogel particle incorporated on average 588 ± 62 cells (Figure S5, Supporting Information). The hydrogel array slide was immersed in cell culture medium supplemented with 1% of CaCl2 for a few seconds, leading to the immediate detachment of hydrogel particles (Movies S4–S10, Supporting Information). CaCl2 was added to the medium to keep the hydrogel particles from dissociating. The medium supplemented with calcium was exchanged every 2 d. Square hydrogel particles whose sides measured 1 mm were also fabricated using this method (Figure S6, Supporting Information). The viability of cells cultured in freefloating hydrogel particles was monitored for up to 7 d via propidium iodide staining and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Figure 3b,c). These results were compared with the viability of HeLa-GFP cells cultured in a standard petri dish (2D positive control) and cells cultured in bulk 2 mm thick alginate hydrogel layers prepared in a 24-well microtiter plate. Already 24 h after the onset of culturing, the viability of cells in freestanding hydrogels was more than 20% higher than that of the bulk hydrogels. The toxicity of cells cultured in the bulk hydrogel rose to 60% after 5 d and 80%–90% after 7 d of culturing, while that of cells in the hydrogel particles rose to just about 20% and 30%, respectively. This is attributable to the cells' superior accessibility to oxygen and nutrients when cultured in the thin freestanding hydrogel particles compared with the limited nutrient and gas exchange in the bulk hydrogel system. The difference in cell viability can be explained by much higher surface area-to-volume ratio of freestanding hydrogels (≈11) compared to bulk hydrogel (≈0.9), which reflects in exchange rate of nutrients and oxygen. One property of ionically cross-linked alginate hydrogel particles is that they dissolve when cultured in standard Ca-negative culture medium. This happens due to the release of calcium ions into the surrounding media caused by the exchange with monovalent cations (such as sodium ions present in the medium).[34] This could be either an advantage (enabling cell release from the hydrogel) or a disadvantage (when long-term cultivation is required). To address this problem, we supplemented the culture medium with 1% (v/v) solution of CaCl2 (Figure S7, Supporting Information). Those results revealed no significant differences in the cells' viability, whereas the viability of cells cultured in cell culture medium (without calcium addition) dropped to 70% of viability on day 7. Thus, the addition of calcium ions to physiological media promotes mechanical integrity without affecting cellular viability.

Magnetic field has been used in various cellular applications, cell sorting, 3D cell cultures, tissue engineering, local hyperthermia therapies, and clinical imaging applications.[35–38] Using the droplet microarray method, freestanding hydrogels can be loaded with functional magnetic particles to enable the use of magnetic field for the remote manipulation of the hydrogels (Figure 4). Magnetic beads measuring 2 μm were added to alginate solution containing living Hela-GFP cells. The viability of cells cultured in freefloating magnetic hydrogel particles was evaluated for up to 7 d using propidium iodide staining, followed by fluorescence microscopy (Figure 4b). MTT colorimetric assay was performed separately (Figure 4c). We observed no significant differences in the viability of the cells cultured in the presence of magnetic beads in comparison to 2D cell culture (Figure S8, Supporting Information). After 1 d of cell culturing
in a 2D and 3D environment, viability remained at 100%, dropping to 70% on day 7.

Magnetic hydrogel particles can be useful for the modular tissue engineering due to the possibility to manipulate both single particles and particle assemblies using external magnetic field. Thus, Movie S11 (Supporting Information) shows the possibility to remotely control exchange of the medium, collection of hydrogel particles, and active movement of particles across culture medium. On the other hand, application of the external magnetic field to a suspension of freestanding magnetic hydrogel particles permits rapid assembly of the particles into stable macroscopic 3D architectures (Figure S11 and Movies S12, S13, Supporting Information). The ability to remotely modulate the density and interparticulate volume of 3D hydrogel architectures has been also demonstrated in a proof-of-concept experiment (Figure S11 and Movie S13, Supporting Information). We showed that this method could be used to remotely compress or expand 3D hydrogel particle assemblies (3 mm diameter, \( n = 250 \)), which is important, for example, for achieving active perfusion of large 3D hydrogel particle assemblies with medium for long-term cell culturing (Figure S11 and Movie S13, Supporting Information). The remotely controlled compression-expansion cycles could be repeated at least 50 times (Figure S11, Supporting Information). Finally, the ability to remotely compress hydrogel particle assemblies was applied to demonstrate the stimuli-responsive release of a small molecule drug incorporated inside the hydrogel using external magnetic field as the stimulus (Figure S9, Supporting Information).

In order to demonstrate the ability to use hydrogel particles of diverse geometries for shape-coding to distinguish between hydrogels’ different compositions, we prepared two types of freestanding hydrogels: round 3 mm hydrogel particles encapsulating HeLa-GFP cells and square 3 mm hydrogel particles incorporating MLTy-CMV-mCherry-neo cells expressing cherry fluorescent protein (Figure 4e). The density of both cell types inside the distinctly shaped hydrogel particles was set to \( 6 \times 10^5 \) cells per mL of alginate solution. The same amounts of freestanding hydrogel particles were mixed in a petri dish. The fluorescence images show that no cross-contamination occurred between hydrogels, and that the cells remained immobile inside the particles (Figure 4). It is also evident that the shape difference opens the way to differentiate between particles’ various

Figure 3. a) Representative brightfield and corresponding fluorescence images of freestanding hydrogels encapsulating HeLa-GFP cells for up to 7 d of culturing time. Dead cells are visualized using PI staining. Scale bar: 1 mm. b) Percentage of live cells obtained using image-based analysis and c) MTT colorimetric assay. Statistical differences by time point analysis were marked with (\( \ast \)), (\( \ast \ast \)), (\( \ast \ast \ast \)), which stand for \( p \)-values < 0.05; \( p < 0.01; p < 0.001 \), respectively. Statistical differences related to the previous time points are indicated by ## (\( p < 0.01 \)) or ### (\( p < 0.001 \)). All results are presented as ± standard deviation.
compositions and encapsulated cell types without special fluorescence labeling.

We have demonstrated a new method to fabricate freestanding hydrogel particles with defined geometries and sizes, while maintaining control of the elastic modulus and the composition of the hydrogel. This platform offers several advantages: (i) thousands of hydrogel particles can be rapidly formed without the need for multiple pipetting; (ii) their size and shape can be controlled using the geometry of the hydrophilic areas; (iii) cells can be encapsulated inside hydrogel particles; (iv) the volume of the hydrogel particles can be in the nanoliter range scale, which is one order of magnitude less than that of a standard well in a 96-well plate; (v) sample cross-contamination is prevented by the use of superhydrophobic barriers; (vi) the hydrogel particles can be used either in the form of an array for screening applications or as freestanding particles. The presented technology was also used to form magnetic hydrogel particles incorporating live cells. These particles...
can be employed for the modular tissue engineering due to the possibility to manipulate both single particles and particle assemblies using external magnetic field. We believe that the ability to easily create and manipulate thousands of hydrogel particles of controlled size and geometry will be essential for 3D cell studies and modular tissue engineering. Finally, such a platform can potentially be applied to other types of hydrogel materials and cells.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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