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

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Abstract

We report a method to rapidly fabricate alginate hydrogel particles of specific sizes and shapes. Our method is based on the formation of arrays of droplets of pre-hydrogel solutions on superhydrophobic-hydrophilic patterns using the process of discontinuous dewetting, followed by their gelation via the parallel addition of CaCl$_2$ to the individual droplets via the sandwiching method. We demonstrate that viability of living cells incorporated within the hydrogel particles is higher during the long-term cultivation than in the case of cells cultured in the bulk three-dimensional hydrogel matrix. Incorporation of magnetic particles into the free-standing hydrogel particles containing living cells enabled ease manipulation of the particles using an external magnetic field.

Introduction

Hydrogels are hydrated three-dimensional (3D) cross-linked polymers resembling natural extracellular matrix (ECM) that provide soft 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, whereas bottom-up approaches aim to generate larger tissue constructs via the assembly of smaller building blocks (usually cell-laden hydrogels) which mimic the in vivo tissue structure of repeating functional units.

The limitations of bulk hydrogels are that they usually lack the hierarchical architecture of in vivo 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.

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

However, there are still too few methods allowing for the fabrication of hydrogel particles with different geometries and dimensions and compatible with the cell encapsulation. New approaches combining the encapsulation of cells into structures with complex geometries and long-term cell analysis 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. 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. Micro-patterning and microfluidic systems have been used to create hydrogels with spatially controlled organization as well as numerous cell-material or cell-cell combinations to analyze distinct biological issues. Micro-patterned arrays have been generated by immobilizing hydrogels on different micro-domains within a plane surface, and they have been used for disease diagnosis, prognosis, biochemical analysis, and therapeutic regimes, and have become an alternative approach for high-throughput multiplexed assays. Strategies avoiding such 2D fixation of various hydrogels have employed shape-coded hydrogel particles as a suspension microarray format for multiplexed bioassays.

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 pre-hydrogel droplets. We applied this method in three demonstrations: (1) preparing an array of hydrogel particles and free-standing hydrogel particles with distinct geometries and sizes defined by the photomask features; (2) examining the cells' in situ viability encapsulated into free-standing hydrogel particles, and (3) constructing magnetic responsive hydrogel particles for modular tissue engineering and shape-coded free-standing hydrogels of distinct cell types.

Results and discussion

To produce the SH-SL array, a nanoporous poly(2-hydroxyethyl methacrylate-co-ethylene dimethacrylate) (HEMA-EDMA) polymer layer was formed on a microscope glass slide followed
by modifying the polymer surface with alkyne groups via esterification.\textsuperscript{20} Afterwards, 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\textsuperscript{20} 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 (SL) areas separated by superhydrophobic (SH) borders. The porous SH regions possessed advancing ($\theta_{\text{adv}}$), static ($\theta_{\text{st}}$) and receding ($\theta_{\text{rec}}$) WCAs of 173°, 170° and 164°, respectively, while the SL areas showed WCAs close to zero.\textsuperscript{20}

Figure 1. Schematic representation of the DMA 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 pre-hydrogel solution on a superhydrophobic-superhydrophilic array. Step 2:
Crosslinking of alginate droplets by performing parallel addition of CaCl$_2$ solutions into the individual droplets via the sandwiching method. By changing the position of the slide 1 (bottom vs top) containing CaCl$_2$ droplets, it is possible to form either an array of fixed hydrogel particles (Step 2a) or detach hydrogel particles to form free-floating hydrogel particles (Step 2b). Scale bar: 2 mm.

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$^{33}$ (Figure 1, Step 1) (Movie S1 and Movie S2). 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.$^{18}$ 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, S2) with droplet height of 14.2 ± 1.3 µL (Figure S1)$^{22}$.

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 CaCl$_2$ solution (1 mol/L) as a crosslinker. The second slide (Slide 2) contained cells suspended in 2 mg/mL of alginate solution. Afterwards, 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 sec) after the addition of CaCl$_2$ 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$_2$ 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 solution.
The stability of hydrogels-on-chip was tested for 7 days of culturing fully immersed in medium, which 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). During the sandwiching step, the DMA slide containing alginate droplets is placed at the bottom. In this case, hydrogel particles could be easily detached from the DMA slide by immersing in culture medium or buffer. Free-standing hydrogel particles containing cells could be also formed using this method (Movie S4). We hypothesize that the reason for the different gelation and adhesion strengths of the hydrogel particles is caused by fast consumption of CaCl$_2$ during the crosslinking 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).

Figure 2. Microscopic images of free-standing hydrogel particles (bright-field images) containing magnetic beads and arrays of fluorescent hydrogel particles anchored to the patterned surfaces (insets). Fluorescent hydrogel particles contain 0.25 wt % of rhodamine 6G as additive. The following geometries were used: squares with 3 mm (a) and 1 mm (b) side length, circle with 3
mm (c) and 1 mm (d) diameter, hexagon of 3 mm (e) and 2 mm (f) side length, triangle with 3 mm side length (g), hearts with width of 3 mm (h) and stripes of 1 mm width. Scale bar 1 mm.

Both free-standing and on-array hydrogel particles of different sizes and geometries could be prepared (Movies S5-8). 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 free-standing hydrogels proved the homogeneous size distribution of hydrogel particles of different shapes (Figure S2).

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 – 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 of cells or cell encapsulation. For example, the shape and size of particles will determine the number of cells per particle. The shape of particles will be important for constructing macro assemblies with diverse architectures, inter-particle 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 multi-particle assemblies for modular tissue engineering. 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 \textit{in-vivo} cellular environment and for achieving higher cell viability during long-term cell culture. The stiffness of hydrogels depends on the concentration of CaCl$_2$ used for gelation. Dynamic mechanical analysis (see supplementary information and Figures S1e-f for details) was used as a non-destructive technique to characterize the viscoelasticity of the hydrogels fabricated using different concentrations of CaCl$_2$. To check the long-term stability of free-standing 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 CaCl$_2$ solution for 1, 3, 5 and 7 days (see Figure S3 for details). Our results revealed that after immersion of crosslinked alginate hydrogels into cell culture medium the hydrogels' storage modulus decreased from 95 kPa (day 1) to approximately 19 kPa on day 7 (Figure S3a). This could be explained by ionic exchange between calcium and monovalent ions (such as sodium ions). However, this tendency seemed to be reversed by a possible continuation of the crosslinking, by adding calcium ions to the medium. The hydrogels' storage modulus increased with the duration of immersion in the calcium-supplemented medium, to approximately 200 kPa, after 7 days of immersion (Figure S3b). Moreover, in hydrogels immersed in calcium-containing medium, the loss-factor values did not vary significantly with time (Figures S3c-d).

In the next step we also fabricated free-standing hydrogel particles incorporating living cells (Figure 3a). We first demonstrated that alginate and CaCl$_2$ exerted no cytotoxic effect on live human cervical tumor cell line expressing GFP (HeLa-GFP) (Figure S4). 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 of alginate to form an array of separated droplets. CaCl$_2$ 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, free-standing hydrogel particles particles of 3 mm diameter at the base. Each hydrogel
particle incorporated on average 588 ± 62 cells (Figure S5). 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). CaCl₂ was added to the medium to keep the hydrogel particles from dissociating. The medium supplemented with calcium was exchanged every 2 days. Square hydrogel particles whose sides measured 1 mm were also fabricated using this method (Figure S6). The viability of cells cultured in free-floating hydrogel particles was monitored for up to 7 days via propidium iodide staining and MTT 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 24h after the onset of culturing, the viability of cells in free-standing 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 days and 80-90% after 7 days 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 free-standing 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 free-standing hydrogels (app.11) compared to bulk hydrogel (app. 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). 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.

Figure 3. a) Representative brightfield and corresponding fluorescence images of free-standing hydrogels encapsulating HeLa-GFP cells for up to 7 days 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 (*), (**), (***) 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.

Magnetic field has been used in various cellular applications, cell sorting, 3D cell cultures, tissue engineering, local hyperthermia therapies, and clinical imaging applications. Using the
droplet microarray method, free-standing hydrogels can be loaded with functional magnetic or other nanoparticles to enable the use of magnetic field for the remote manipulation of the hydrogels (Figure 4).

Figure 4. Magnetic free-standing hydrogel particles fabricated using the droplet-microarray approach. a) Representative brightfield and correspondent fluorescence images of magnetic hydrogels encapsulating HeLa-GFP cells and magnetic particles for 7 days of culturing time. Live and dead cells are represented by green (GFP) and red (PI) color, respectively. Scale bar: 200 μm. b) Percentage of live/dead cells using image-based analysis (cell counting) and c) MTT colorimetric assay. Absorbance was read at wavelength of 570 nm. All results are presented as ± standard deviation. d) (I.) An array of square-shaped magnetic hydrogel particles (3 mm size length), (II) free-standing hydrogel particles formed by immersion the array in buffer, (III) manipulation of the particles by pipetting or (IV) an external magnetic field. e) Representative images of (i) brightfield, (ii) fluorescent and (iii) overlay of co-culture hydrogels with circle and
square shapes. HeLa cells expressing GFP cells (green) were immobilized in circle-shaped free-standing hydrogels and MLly-mCherry cells (expressing fluorescent red) were immobilized in squared-shaped free-standing hydrogels. Scale bars: 1 mm.

Magnetic beads measuring 2 μm were added to alginate solution containing living Hela-GFP cells. The viability of cells cultured in free-floating magnetic hydrogel particles was evaluated for up to 7 days 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). After 1 day 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 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 free-standing magnetic hydrogel particles permits rapid assembly of the particles into stable macroscopic 3D architectures (Movie S12, S13 and Figure S11). The ability to remotely modulate the density and interparticulate volume of 3D hydrogel architectures using external magnetic field has been also demonstrated in a proof-of-concept experiment (Figure S11, Movie S13). 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, Movie S13). The remotely controlled compression-expansion cycles could be repeated at least 50 times (Figure S11). 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 an external magnetic field as the stimulus (Figure S9).

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 free-standing 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 free-standing 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 compositions and encapsulated cell types without special fluorescence labeling.

Conclusions

We have demonstrated a new method to fabricate free-standing 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 composition 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 free-standing 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 particles 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 different types of biomaterials, hydrogels and cells.

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