Directed Self-Assembly of Complex-Shaped Microtissues

The field of tissue engineering has given rise to a new generation of tools that offer precise control over the structural organization of groups of cells or even individual cells, allowing the design of in vitro tissues that more closely resemble their native counterparts. Beyond evaluating structure and function, research now is focused on the function of structure.

Cells traditionally cultured on flat petri dishes or flasks show morphologies and behaviors that can differ significantly from those of cells in the in vivo setting. Tissue engineering has developed newer methods that allow the culture of cells in three dimensions.

Most of these methods create 3D tissues by seeding cells onto a scaffold of naturally occurring proteins (e.g., collagen) or biodegradable synthetic polymers (e.g., *poly*-lactic acid), and these scaffolds can be formulated in different manners, such as porous sponges or water-laden hydrogels. Numerous studies have used these cell/scaffold combinations in vitro as model tissues and in vivo as replacements or substitutes for diseased or damaged tissues.

For some time, biologists have known that in the absence of a petri dish, scaffold, or adhesive substrate, cells will interact with one another to assemble spontaneously into a 3D microtissue or spheroid. This is an intrinsic biological property of cells.

Through the years, two methods have been used for the self-assembly of spheroids from monodispersed cells: spinner culture and hanging drops. In the spinner culture method, monodispersed cells kept in constant motion collide with one another and form multicellular spheroids. For the hanging drop method, a very small volume (~50 μL) of monodispersed cells is pipetted onto a surface, such as the lid of a petri dish, and this surface is inverted so that the drop hangs under its own weight. The cells within this drop settle to the bottom of the drop, where they contact one another and undergo self-assembly into a spheroid structure.

These methods facilitate self-assembly, because the cells are combined in an environment that favors cell-to-cell as opposed to cell-to-substrate or cell-to-scaffold interactions. They are scaffold-free and entirely cellular. When the spheroids are sectioned, their cell morphologies and tissue architecture closely resemble those of organs.

The cells within spheroids have been shown to differentiate and to have increased in vivo-like gene expression as compared to monolayer cultures. Spheroids of cardiomyocytes beat; hepatocyte spheroids develop bile canaliculi. Extracellular matrix proteins are produced and organized by the cells themselves, as it is done in the body, and cell-to-cell interactions dominate throughout the structures. It has been clear that organization of cells in 3D clusters is vital to optimal cellular function.

From a thermodynamic perspective, it has been thought that when cells spontaneously self-assemble into a 3D microtissue (such as small drops of oil spontaneously aggregating in water), the resulting structure would minimize the surface-area-to-volume ratio. A sphere or spheroid is just such a structure. Moreover, the spheroid microtissue has also been proposed as a building block for construction of even larger, more complex engineered tissues.

But what if the self-assembly of microtissues was not limited to the spheroid? What if complex-shaped microtissues could be self-assembled directly from monodispersed cells?

Recently, we developed a method for self-assembling 3D microtissues of prescribed complex branching geometries, showing that the tissue engineer "architect" may not be limited to the spheroid as the only building block. In this method, nonadhesive (i.e., agarose) hydrogels were cast from micromolds designed in computer-aided design (CAD) software and produced by rapid prototyping. The micromolds were designed such that the hydrogels have cell "seeding chambers" that contain any number of recesses of any shape desired.

When a suspension of mono-dispersed cells is pipetted onto the hydrogels, cells sink to the bottoms of the recesses over minutes. The bottoms of the recesses are rounded, causing cells to contact one another, and the agarose is nonadhesive for cells, thus both aspects promote the requisite cell-to-cell interactions critical for self-assembly.

The key is that because recess architecture can be designed and the gels are nonadherent to cells, the self-assembly process can be

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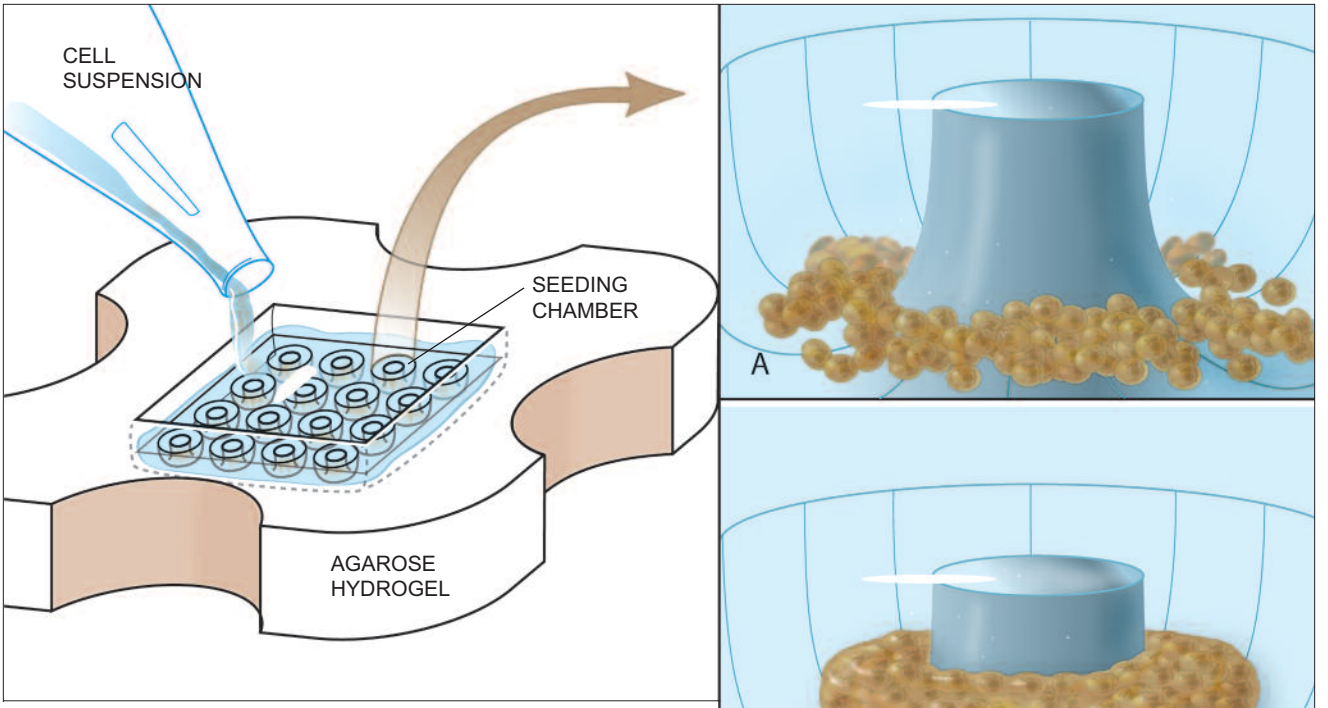

Directed self-assembly of complex-shaped 3D microtissues. Monodispersed cells are seeded onto the surface of a micromolded, nonadhesive agarose hydrogel equilibrated in cell culture medium. Cells sink to the bottom of the recesses (*panel A*) and do not stick to the agarose, but contact one another and self-assemble into a 3D tori microtissue (*panel B*) .

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organized spatially such that complex shapes are formed. We call this process *directed self-assembly*.

In a recent study, we used this method to test the abilities of two different cell types to self-assemble into complex-shaped microtissues. As agarose is translucent and the hydrogels are thin enough for microscope viewing, we also were able to evaluate the kinetics of the self-assembly processes.

We used normal human fibroblasts (NHFs) and H35s, a rat hepatocellular carcinoma cell line, to determine if cells could self-assemble into complex-shaped microtissues. The two cell types were seeded onto micromolded agarose gels with rod, tori, and honeycomb-shaped features. These shapes were simple step-ups in complexity from the spheroid but introduced important architectural differences to the self-assembly process and new challenges to the cells.

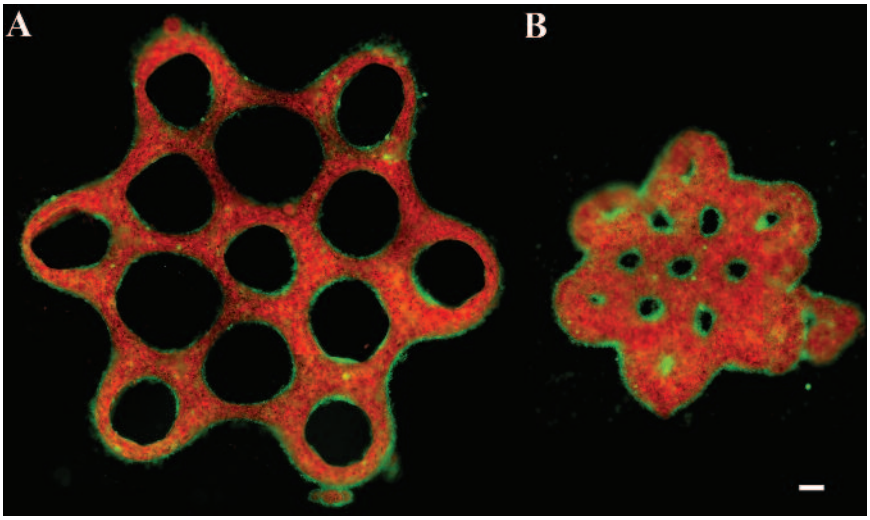
Rods are simply unconstrained spheroids elongated in one dimension and are formed by seeding cells in trough- shaped recesses. Tori-shaped microtissues are essentially rods wrapped around a central peg of nonadherent agarose. Honeycombs are a network of interconnected rods and tori.

The main limitation on design was the diffusion distance of nutrients in, and waste metabolites out, so as to avoid the formation of necrotic cores (a thickness reported to be 250-300 µm). Thus, recesses were designed to generate microtissues of about 300 µm thicknesses in all dimensions.

We found striking differences in the self-assembly behaviors of the two cell types. In the rod assay, cells would initially coat the bottoms of the trough-shaped recesses. As self-assembly proceeded, cells would begin to rearrange from several-layer-thick sheets into 3D rounded rods. H35s consistently formed rods that were about 50% of the original trough length, suggesting that there was a balance between trough length, weight of the rods, and forces driving H35s self-assembly toward spheroids. In contrast, NHFs always contracted to spheroids in trough recesses in a very rapid process. Both H35s and NHFs formed tori, but H35s tori were significantly thicker and more stable.

Consistent with the rod and tori data, only H35s could maintain honeycomb shapes for extended periods of time. Interestingly, H35s honeycomb architecture was maintained even when the microtissues were removed from micromolds and allowed to sit on nonadherent, agarose-coated plates. This suggests that complex-shaped microtissues could be harvested for use in other applications, such as building larger structures.

When fluorescently labeled NHFs and H35s were mixed and seeded into the micromolds, the cell mixture formed a complex-shaped microtissue and also spon-



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Self-assembled honeycomb microtissues. A mixture of labeled NHFs (*red*) and H35s (*green*) formed a honeycomb microtissue 24 hrs after seeding. Honeycomb microtissues are shown in the micromold (*panel A*) and then out of mold and relaxed for 6 hrs (*panel B*). Scale bar = 200 mm.

taneously self-sorted. The NHFs formed the core of the microtissue, and the H35s formed the outer coating of the microtissue.

Self-sorting of mixed-cell populations has been documented by others with spheroids, but in our complex shapes, we observed an additional phenomenon: self-sorting was influenced by the geometry of the microtissue. In tori, the NHFs formed a continuous tori in the central core of the microtissues, but it was not located in the perfect center of the tori microtissue. Instead, it occupied a position closer to the agarose peg than to the outer edge of the tori. Likewise, the H35s formed the outer coating of the tori, but this coating was thin on the surface contacting the agarose peg and thicker on the surface distal to the peg.

In honeycombs, this same pattern was repeated around the outermost agarose pegs of the micromold. However, in the center of the honeycombs where the microtissue was suspended away from the pegs in all directions, NHFs occupied a more centrally located and evenly distributed core position. This suggested that recess geometry could influence relative positions of the two cell types within the microtissues.

Interestingly, the mixed microtissues had stabilities and rates of self-assembly more like NHFs alone, suggesting that NHFs were dictating microtissue behavior from an internally sorted position. It was also clear that microtissues that contained NHFs were under significant tension. Tori-shaped microtissues containing NHFs could squeeze their central pegs and would contract their lumen if released from the pegs. Mixtures of NHFs and H35s would form honeycombs, but the presence of NHFs caused tension that made the mixed honeycomb pop off the micromold. The role of cell-mediated tension on self-sorting in 3D and the stability of the microtissue shape is an exciting new area.

These results show that self-assembly of 3D microtissues is not restricted to the spheroid, but that complex-shaped microtissues can be produced via directed self-assembly. Furthermore, different cell types (NHF vs H35s) can have distinct influences on mixed microtissue structure and behavior depending on the geometric parameters under which directed self-assembly occurs.

Directed self-assembly enables the easy and rapid formation of 3D microtissues of any shape and size. The tissue engineer simply designs the micromold and adds monodispersed cells. The cells do the rest and spontaneously form the 3D microtissue.

We are just beginning to understand the biological rules governing this self-assembly process, and in addition to cell surface adhesion/cohesion events, we are beginning to understand the role of cell-mediated tension on this process. This ability to generate complex-shaped microtissues with efficiency introduces the possibility of developing tissue-like constructs more easily from complex-shaped building blocks as opposed to spheroids.

We also have found that numerous other cell types can self-assemble complex shapes in 3D, extending the method to a variety of tissues. With directed self-assembly, future studies can begin to evaluate the effect of 3D microtissue architecture on cellular function.

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Pub date: 5 Feb 2008

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