Organ Printing: Self-assembling Cell Aggregates as "Bioink"

Self–assembly, perhaps the most fundamental mechanism in the origin of life and the evolution of complex biological organization, manifests itself at all scales in living systems. To grasp a specific biological structure, we must be able to disassemble and reassemble it. Only by demonstrating our ability to do so can we claim that our understanding of the rules and principles governing the functioning of tissues and organs is complete.

Up to now the main direction in tissue engineering was based on using biodegradable scaffolds as spatial support for the assembly of isolated cells into tissues. It was assumed that the scaffold would provide the right conditions to engineer organs with desired shape and mechanical properties. Seeded cells would proliferate, produce extracellular matrix, and eventually replace the scaffold to form living tissues or organs. This process is long, and cell survival may be an issue.

Self-assembling cell aggregates may provide a better starting point and faster organ formation. Such aggregates are already a popular tool for studying cell adhesion, cell sorting, and tumor growth, and they also have been used in drug testing. It recently was suggested to use cell aggregates to build tissue-engineered cartilage, retina, liver, and other organs.

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An intriguing idea is to employ cell aggregates as building blocks in the emerging technology of organ printing. Organ printing is an application of rapid prototyping or computer-aided layer-by-layer deposition in tissue engineering. Instead of seeding isolated cells, aggregates could be printed directly on the surface of stimuli-sensitive gels. Addition of another layer of gel is followed by the printing of another layer of aggregates and so on. After fusion of aggregates, tissue constructs of the desired geometry may be formed. The gel is subsequently eliminated.

How are aggregates made, and what are the criteria for ideal cell aggregates?

Cell aggregates may be fabricated by several methods. The classic "hanging drop" method is the simplest (cells in an inverted drop of tissue culture medium precipitate and form a sphere). Shaking cell suspensions in appropriate laboratory flasks is another popular approach. Many modifications of these basic techniques also exist.

Our experience demonstrates that the most effective way of making aggregates of controlled size is to centrifuge the suspension and then cut the resulting pellet into similar-sized fragments. (Cutting has recently been automated to produce identical cylinder-shaped pieces with an aspect [diameter to length] ratio of one.) Subsequent incubation in a gyratory shaker leads to the rounding of fragments into spheres.

Controlling the size of aggregates is critical for their effective deposition using automatic cell dispensers or cell printers. Aggregate size is limited by the capacity of nutrients to diffuse to the central cells and depends on cell type. Aggregates typically cannot exceed 500 μm in diameter.

The ideal shape of the aggregate is spherical to avoid the potential clogging of nozzles. The cohesivity of the aggregates depends on the nature of their constituent cells. It can also be affected by the physical and chemical properties of the employed gel.

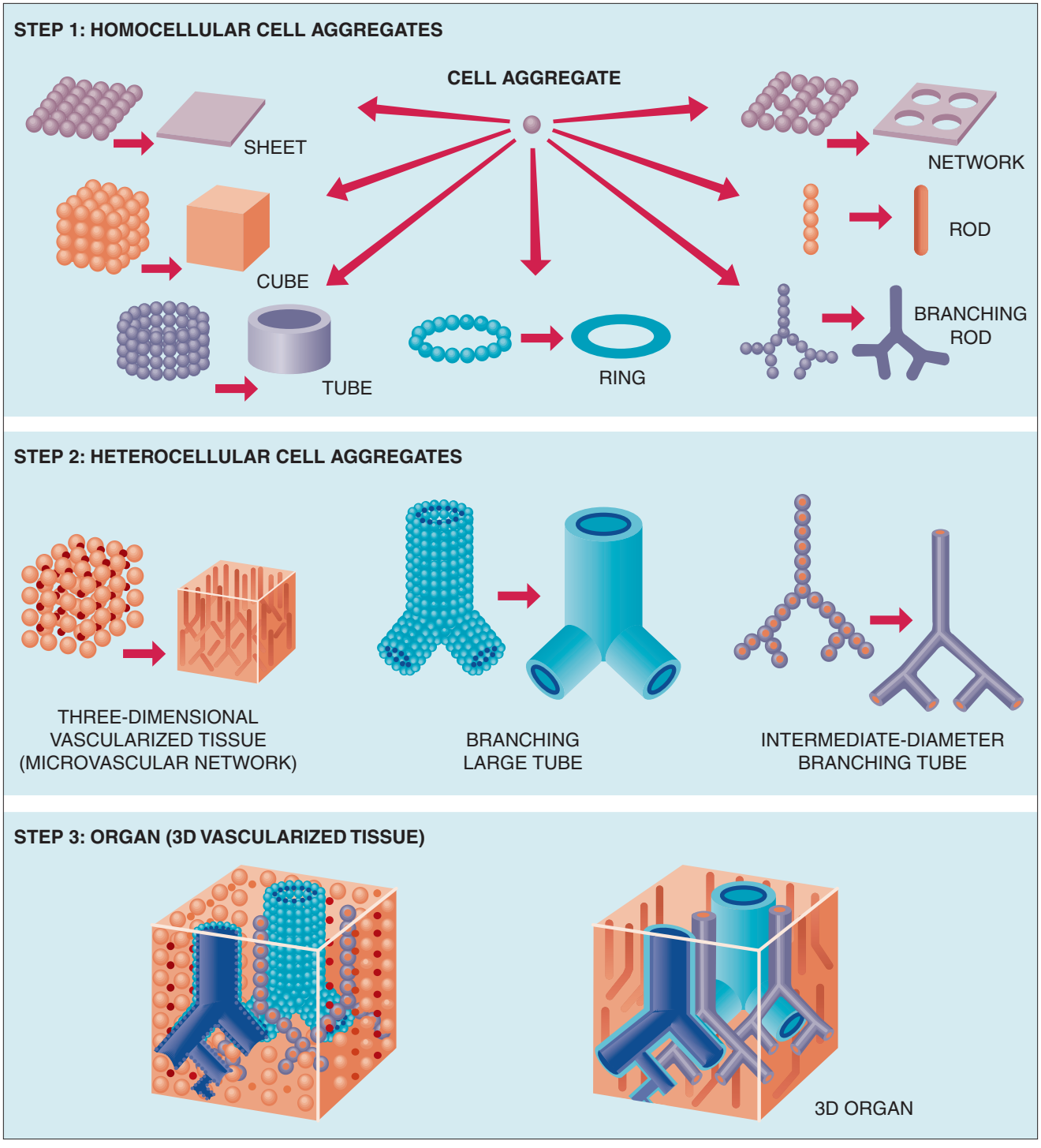
The composition of aggregates can be varied from homocellular to hybrid heterocellular, and they can be fabricated to contain extracellular matrix in desired quantities. Finally, cell aggregates can have elaborate internal structures with complex pre-built branching or interconnecting architecture.

Thus, the ideal aggregate characteristics are determined by the constraints imposed by the applied printing technology and the specific intrinsic properties of the employed gel and tissues or organs to be printed.

What are the advantages of using cell aggregates for organ printing?

According to calculations, three-dimensional printing of an entire organ such as a kidney would take no more than 2 hours, even if single cells were employed as printing units (drops). Using aggregates composed of thousands of cells can reduce printing time by a factor of 10 or more. Reduced printing time enhances cell survival. The harsh mechanical conditions during passage through the printer's nozzle are less damaging for aggregates than individual cells. However, the death of some cells in the aggregate during the process is not detrimental for the final outcome.

Aggregates provide critical cell concentrations, which are difficult to achieve by other methods. Finally, because aggregates containing



Cell aggregate printing. Homocellular aggregates can be used to form simple building blocks, which then are combined with other cell aggregate types to fabricate simple vascular structures. Cellular aggregates can be fabricated to contain extracellular matrix and pre-built branching or interconnecting structures to produce complex vascularized tissues (organs).

several cell types or a pre-built internal structure are easy to engineer, considerable time can be saved during post-printing tissue and organ maturation.

How are cell aggregates formed in three dimensions, and what are the criteria for an ideal gel?

The critical property that makes the use of cell aggregates in organ printing possible is their fluidity. Because fluids can flow, aggregates can fuse into more complex structures, as has been proven experimentally. This leads to the next level of biological organization.

Thus, in the context of organ printing self-assembling cell aggregates can be employed as "bioink," like the ink used in ink-jet printing technologies. The potential appli-

cation of such self-assembly-based bioink has recently been reported: aggregates placed contiguously along a ring in an appropriate three-dimensional gel fused into doughnut-like structures.

We used aggregates with fluorescently labeled cells to follow structure formation. Cell sorting and the remodeling of extracellular matrix by the cells were found to be crucial mechanisms during pattern evolution.

Cells in aggregates placed in three-dimensional gel have a difficult choice between the competing processes of cell-cell and cell-substrate adhesion. Experiments indicate that if the aggregate is too cohesive, cells cannot migrate, whereas if the aggregate is not sufficiently cohesive, cells disperse into the gel. No fusion takes place in either case.

Thus, to achieve fusion and structure formation, a gel with optimal properties has to be designed for the particular cell types or their mixtures.

It is obvious that the gel must be nontoxic, biocompatible, and biodegradable. It must have the capacity to solidify in response to specific stimuli in a controlled environment. The gel could be thermo-sensitive, photosensitive, pH-sensitive, or even molecule-sensitive. It also must be sufficiently fluid to be printed through the relatively thin nozzles. Cell-gel adhesion must be controlled, e.g., by the addition or incorporation into the gel of RGD fragments or other peptides. The sol-gel transition has to take place after the delivery of aggregates.

Thus, aggregates must survive printing and the process of crosslinking, and methods triggering it must be nontoxic. The capacity of the gel to undergo fast and nontoxic solidification is its most critical property for organ printing technologies.

The ideal gel for organ printing that fits all the desired criteria does not yet exist. However, the feasibility of synthesizing and designing such intelligent or smart gels has already been shown. Because the number of gels with particular properties is growing rapidly, a breakthrough in this field is to be expected.

What are the most likely potential applications of organ printing?

The first application of this technology will be the printing of three-dimensional tissue constructs based on high through-put screening assays (e.g., printing aggregates composed of endothelial cells and angioblasts, according to input from vasculogenesis or angiogenesis). Such constructs could be used for drug screening, identification of genes and their functions, as well as for personalized medicine (e.g., testing the relative efficacy of anti-tumor drugs in three-dimensional structures printed from the patient's tumor cells).

Finally, it is not hard to predict that avascular tissues and organs (such as nose, ear, skin, or large-diameter blood vessels) will be printed first. Printing of complex vascularized organs such as heart, liver, or kidney will be possible only after whole intraorgan vascular trees with harmonically branching large vessels (arteries and veins), intermediate vessels (small arteries, arterioles, small veins and venules), and microvasculature (capillaries and sinusoids) have been printed. Without a branching vascular tree, the printed organ cannot be effectively perfused and would not survive.

Thus, the first and most critical step is printing of three-dimensional tubes. In experiments, we were able to form three-dimensional tissue tubes from aggregate rings placed contiguously on top of one another, with the fusion of the aggregates in both the horizontal and vertical directions. This shows that printing of large-diameter vessels is feasible. Several groups have already demonstrated that branching microvascular networks in contiguous gels can be created reproducibly.

The difficult part of organ printing is creating intermediate vessels, which are too small to be formed by direct printing of cell aggregates and too large to be formed by simple enlargement as in the case of microvasculature. Additional technology, such as laser-based ablation-induced apoptosis, cell sorting, and more complex cell aggregates, could be employed to form lumenized intermediate blood vessels.

At least three effective ways exist to integrate printed parenchymal tissue with a printed vascular tree. Aggregates composed of parenchymal cells of a specific organ (cardiomyocytes, hepatocytes, or kidney cells) could be printed simultaneously with aggregates formed by endothelial cells or angioblasts. It is possible to employ composite aggregates with parenchymal and endothelial cells in the interior and periphery of the spheroid, respectively. Finally, the printing of parenchymal cell aggregates with a pre-built, internal, three-dimensional microvasculature is another possibility.

Thus, self-assembling cell aggregates or "bioink" is a critical and essential element of the evolving organ printing technology.

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