TechNews

PRINT ON DEMAND

Advances in 3-D printing and biomaterials are helping to shape the field of regenerative medicine. Sarah Webb examines the latest developments.

Credit: L. France at Cornell University.

Three years ago, Long Island, NY ear, nose, and throat surgeons David Zeltsman and Lee Smith were searching for a way to treat patients' tracheal damage. Their main problem was that cartilage in the body doesn't readily regenerate and is difficult to culture in a 3-D matrix, so they needed to find a new source of tracheal tissue. It was then that the two physicians started to wonder if it might be possible to 3-D bioprint implantable tracheas.

They consulted Daniel Grande's laboratory at the Feinstein Institute for Medical Research in Manhasset, NY, where researchers were already using 3-D printing to build load-bearing joint cartilage. After early discussions, Todd Goldstein, then a graduate student, decided to take the lead on the trachea project, leveraging his knowledge and skills in biology, engineering, and computers. Goldstein collaborated with another lab to learn how to work with epithelial cells, and within a year, the team had a prototype tracheal tissue. Animal studies showed that the printed tracheal tissue was just as strong as the tracheal tissue found in mammals (1). The researchers are now trying to bring printed tracheas to the clinic, Goldstein says. "Ideally, it'd be nice to do this in the OR on demand."

The Long Island group is not alone in turning to bioprinting—in fact they are only one of a growing number of interdisciplinary teams that are pairing a knowledge of stem cells and cell culture with 3-D printing techniques to solve problems in regenerative medicine.

Precise, reproducible, and scalable

Nearly three decades ago, surgeon Anthony Atala and his team began building novel tissues for regenerative medicine by hand in the laboratory. It was painstaking work, harvesting cells from patients, building structures layer-by-layer, and culturing these handmade scaffolds to produce functional tissues such as skin, bladder, and cartilage.

Although they tested some of these handmade tissues in the clinic, the approach was far too slow and laborious to treat large numbers of patients. So 14 years ago, Atala's team, now located at the Wake Forest Institute for Regenerative Medicine in Winston-Salem, NC, began to remodel standard desktop inkjet printers to spit out cells. "It really was an automation strategy," he says. "You're getting three things. You're getting precision. You're getting reproducibility, and you're getting scalability."

The precision comes from the use of small nozzles to control the placement of cells and "inks," the biomaterials printed around the cells that form a structural framework and also provide nutrients and growth factors. Today, a variety of cell printing strategies are available, but two of the most popular techniques are inkjet printing and extrusion-based printing.

Inkjet bioprinting of cells was first demonstrated by Hewlett-Packard in the late 1980s. "It's a more difficult technology," says Brian Derby of the University of Manchester in the United Kingdom. Printed inks must be liquid as they're squeezed through the nozzles in the piezoelectric printing process. But to form and maintain a 3-D structure, those liquid inks must gel quickly as they're deposited. Even with that challenge, Derby finds the technique more versatile than other approaches because inkjets allow for "color" printing: instead of printing red, blue, green, and black, nozzles in a bioprinter might include, multiple cell types, structural biomaterials, and other chemicals in a single manufacturing process.

Thomas Boland of the University of Texas, El Paso, one of the early developers of inkjet cell printing, emphasizes another advantage of the approach—the method is non-contact, thus avoiding cell contamination. As a result, his team routinely employs 15 different printing modules with various combinations of cells and inks.

In extrusion bioprinting, the nozzles function like tiny syringes, squeezing out ribbons of cells and hydrogel. Inks don't have to flow as freely. The hydrogels that encapsulate cells can remain relatively stiff—like toothpaste being squeezed out of the tube—as they're being printed. Bioengineer Lawrence Bonassar of Cornell University uses this strategy for building collagen-based structures. Instead of building layer-by-layer, material is extruded directly. "As soon as the material kind of comes out of the printer and solidifies on the stage, then we're kind of done," he says, and the process gives Bonassar and colleagues full control of the assembly in three dimensions.

Inkjet and extrusion bioprinting each has its pluses and minuses, and the best choice can depend on a combination of tissue size, resolution, and time needed to print a particular construct and optimize cell viability. Jennifer Lewis's laboratory at Harvard University relies primarily on extrusion-based methods, says Kimberly Homan, a postdoctoral researcher in the group. But that's an approach that fits well with the lab's overall design philosophy, she adds. Instead of focusing on printing all the structural details, she and her colleagues are most concerned with setting cells up to proliferate and thrive. "We're trying to constantly ask ourselves, what is the minimum architecture that we could print and then let biology take over from there and self-organize."

Inkjet and extrusion strategies don't have to be mutually exclusive. In 2016, Atala's group published an article presenting their integrated tissue-organ printer (ITOP) system (2). This hybrid strategy includes several nozzles, and the ITOP can print using both the inkjet and extrusion techniques. The researchers demonstrated the use of this technique in building test scaffolds as well as samples of bone, cartilage, and skeletal muscle.

Data associated with this 3-D computer-aided design (CAD) model were also used to create code to print a section of jaw bone.
Credit: Wake Forest Institute for Regenerative Medicine.

A bioprinted jaw bone structure produced in Anthony Atala's laboratory.
Credit: Wake Forest Institute for Regenerative Medicine.

Think "inks"

Even though much of the novel interest in bioprinting comes from the machines themselves, it's the inks that most researchers focus on. These materials must be biocompatible and printable while also possessing physical properties that support the cells and a printed tissue's structure. Researchers also often use these inks as placeholders. "You want the materials to be a guiding mold for the cells to form the tissue," Atala explains. "You're trying to put a scaffolding in that will degrade over time, but as it degrades, the cells lay down their own glue."

Most groups working on tissues rely on combinations of naturally derived biopolymers such as collagen, gelatin, and fibrin for their inks. Alginates are another option, one that Bonassar's group used routinely before recently switching to collagen. Alginates have an ionic phase transition from solid to liquid, which researchers can trigger by adding multivalent cations such as calcium.

Researchers are also exploring chemically modified biopolymers to allow control over the printing process. Ali Khademhosseini's laboratory at Harvard Medical School has combined biomaterials with photocrosslinking groups and an array of digital micromirrors to pattern combinations of printed cells and hydrogels. After hydrogel printing, they can tune the mirror array in a particular pattern and then use light to lock in a design.

But even with natural biopolymers, researchers spend a considerable amount of time optimizing formulations to get the best combinations for each application. "Whenever we think about new projects, one of the first things we do is tailor the inks to the cells that we're printing," says Homan. In addition, researchers can include other chemicals, such as growth factors, in the inks.

Time is also a consideration since many printed structures need to be incubated in bioreactors with careful control of temperature, nutrients, and other factors to complete their development into tissues. Microfluidic devices, such as those developed by Lewis and Khademhosseini, can also provide support for newly printed tissues. Atala and his colleagues typically need a total of 6–8 weeks to transform human cells into a functional tissue with 3-D bioprinting; approximately 2 weeks of that time is just for culturing the new tissue after printing.

Plumbers of biological materials

One of the great challenges when fabricating large 3-D structures with cells is figuring out how to feed them. Nutrients and waste can only diffuse short distances, up to a couple of hundred microns. Therefore, larger 3-D structures need some type of vasculature or other plumbing for cells to survive and thrive.

One approach to creating this needed vasculature is to try to print the capillaries themselves. Boland's group recently used inkjet printing to produce a vasculature grid between traditional collagen layers of skin fibroblasts. Their overall goal is to eventually produce thicker skin grafts that would be suitable for treating chronic ulcers in diabetes patients. Comparing the behavior of this structure with a commercial two-layer product that lacked vasculature in immunocompromised mice showed that overall incorporation and skin contraction in these two tissues were relatively similar, Boland says. But the vascularized graft produced additional microvessels within 2 weeks, which appeared to incorporate human cells. Now the team is trying to determine whether the human cells are the ones that were printed or if they were produced within the mouse (3).

Because of the precision and detail required for printing vasculature, other teams are looking for ways to design large-scale microchannels or vessels while also taking advantage of cell biology by integrating temporary channels into their printed designs, adding growth factors and other cues that prompt the printed cells to develop vasculature on their own. "We print the highways and let the small roads be enabled by the tissue needs," notes Homan.

To figure out how much vasculature they need to print and where they can rely on biology, Derby's group has collaborated with a microsurgeon for studies in knockout mice. They print small spherical globs of gel that look like tiny watermelons and encapsulate a large vein-like printed vessel. When surgically attached within the bloodstream of mice, the researchers then observe these structures to see how smaller blood vessels form. The team has also been working on strategies for installing basic vascular plumbing within larger proto-tissues that could generate networks of smaller blood vessels.

Another approach to building plumbing into a printed tissue construct is to print a channel network using "fugitive" or "sacrificial" inks that wash away after printing. For this, Lewis's group uses Pluronic F-127, a commercially available biocompatible material that's solid at room temperature, but liquefies as it cools. "You can essentially print it right alongside printed cells and then cast another material over it to lock everything in place, and then selectively, just by cooling it down, you can remove the fugitive ink," Homan says. "That innovation has allowed us to become excellent plumbers of biological materials." (Atala has also incorporated this idea in his ITOP strategy.)

Homan and her colleagues generally use these types of microchannels as conduits, which allow them to perfuse their structures with nutrients and flush out waste. Last year, they printed a piece of tissue 1-cm thick cube of tissue that they kept alive on a microfluidic chip for several weeks using this strategy. The structure incorporated a variety of stem cells along with a network of microchannels formed with a fugitive ink.

After removing the ink, Homan's group perfused that network with human umbilical vein endothelial cells that produced, vessel-like structures. Over a period of several weeks, they perfused the cells with nutrients and growth factors and flushed out waste. And over time, the novel tissue supported differentiation of the printed cells into osteoblasts while growing alongside endothelium and fibroblasts (4).

Kimberly Homan develops printed architectures to enable biology and self-organization of cells in the Lewis lab at Harvard University.

Credit: L. Sanders.

Crafting cartilage

Many efforts in regenerative medicine and 3-D bioprinting to date have focused on cartilage. An important target, it's found throughout the body but cannot regenerate itself when damaged, so a range of clinicians including reconstructive surgeons, orthopedists and cardiovascular surgeons would like to have better strategies for repairing and replacing this important tissue.

Replacing cartilage presents different challenges from other tissues. Although culturing cartilage cells in 3-D is challenging, cartilage's structure is somewhat simpler since it lacks blood vessels. But it also requires other components such as organized collagen and proteoglycans to give it mechanical stiffness.

Bonassar of Cornell University has focused on cartilage since his PhD research. Like Atala, he was looking for ways to scale up the manufacture of these regenerated tissues. When he moved to Cornell more than a decade ago, he began collaborating with Hod Lipson, a mechanical engineer who has used 3-D printing and other techniques such as injection molding to construct materials for batteries and robotics. They soon started to collaborate on 3-D printing strategies and recruited a student to help, publishing their first paper together in 2006.

Since then, Bonassar and Lipson have printed many different types of cartilage tissue and bone cells. The tissue they've pro-

duced

An example of a 3-D bioprinted structure, in this case a bioprinted ear.
Credit: L. France at Cornell University.

duced that is closest to clinical use is cartilage for treating a congenital defect called microtia, where children are born without an outer ear structure [5]. Current treatment options for microtia are limited. At 5 or 6 years-of-age, surgeons harvest ribs from these children to obtain cartilage and then reshape that tissue to produce the ear structures. The ribs can't fully heal, and the quality of the results can vary widely from surgeon to surgeon, Bonassar says. But he and his colleagues can take a small cartilage biopsy from an ear, combine that with marrow-derived stem cells, and then use 3-D printing to generate an outer ear structure from elastic cartilage. Within a day, the structure is ready for implant in their animal studies. Bonassar expects that human trials of this tissue could begin within 5 years.

Regulatory challenges

Even though researchers are confident bioprinted tissues and even organs will make important contributions in the clinic, there's a long way to go before doctors will routinely print and implant tissues. A lot of the funding in this area of research is focused on organs on a chip—microchips lined with human cells, Homan says. Those devices will likely make their mark soon for drug development and disease modeling. [More to come on this topic in March, in a feature about 3-D cell culture.]

"We are very cautious of using what we call 'the o-word [organs]," Homan says. "We don't want to overhype the field." She and her colleagues are working toward a functional nephron—1 of just 1.3 million such structures in a kidney. So far, they've constructed two or three subunits of the nephron that work well.

Atala's group is currently working on the initial studies required by the FDA to begin clinical trials of three different types of printed tissues: flat (skin), tubular (blood vessels), and hollow structures (bladder). They previously have implanted structures like these made by hand in patients. Solid structures such as the heart, liver, and kidney are far more complex, Atala notes, because of the sheer number of cells per centimeter and their vascular needs.

A few companies are trying to get niche tissue products launched in the marketplace. For example, a spinoff company from Boland's laboratory, TeVido BioDevices, is developing a replacement for the nipple complex in women who have had a mastectomy to treat breast cancer. But even though there are few clinical alternatives to their approach, development has been tricky. It's complex and highly regulated, and says Boland, "the market is very, very fragmented." And the clinicians who work on skin aren't always the same doctors involved in breast reconstruction, so it can be difficult to get the right combination of people on board.

Researchers will also face a host of production challenges. Labs can culture millions of cells, Homan says, but production scale would require billions of cells under high quality control and with attention to cell compatibility. Better analytical tools are needed for measuring the quality of printed constructs as they get larger to insure that they're growing and developing appropriately and that there aren't any blockages or clots within the tissue vasculature, she adds. Khademhosseini's lab has been working on some of these issues, constructing sensors, mini-microscopes and other tools for monitoring the cell's environment and secretions.

More generally, regulatory agencies worldwide haven't worked out robust procedures for testing that cell based products tailored to individuals are both reliably manufactured and improve patients' health. With other medical devices, there are clearer guidelines for ensuring good manufacturing practices—that a device is made well and made the same way every time. With a tissue-engineered device, it's harder to judge efficacy. "It's not really clear how to judge when something is effective or not, and if it's effective in one patient, we can guarantee the same procedure, or the very slightly different procedure you would have to use for a different patient, is equally effective," says Derby.

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