CELL CULTURE'S SPIDER SILK ROAD

A *Euprosthenops australis* spider in the funnel of its web. Credit: Anna Rising

A number of synthetic and natural materials have been tried in cell culture and tissue engineering applications in recent years. Now **Jeffrey Perkel** takes a look at one new culture component that might surprise you—spider silk.

At the north end of the Utah State University (USU) campus in Logan, Utah, Randy Lewis' lab in the new USTAR Bioinnovations Center is on the cusp of a materials science gold rush.

Lewis' gold isn't hidden deep in the snow-capped peaks just beyond his corner office though; it's buried in the bacteria, silkworms, genetically modified alfalfa sprouts, and even goat's milk found in his lab. These are the recombinant biofactories that Lewis and his colleagues have harnessed to generate what many hope will be a new bioengineering treasure: *spider silk*.

Stronger than steel, yet springy like a green tree branch, biocompatible, biodegradable, and relatively non-immunogenic, the potential applications of spider silk span a wide range of industries. "We're working with people on everything from medical products to sporting goods to tires to almost everything in between," Lewis says.

But first researchers must actually obtain useful quantities of spider silk for testing and product development—and that has not been easy.

A Tangled Web

Unlike silkworms, spiders cannot be domesticated. They are territorial, and they are cannibals. Collecting spun webs, says Lewis, the USTAR Professor of Biology at USU, "is kind of a defeatist proposition," because different silks have

different properties, and mixing them together dilutes the batch. The only way to acquire significant amounts of pure native spider silk is to collect wild spiders and then pull the silk from their bodies, like dental floss.

Many researchers, including Lewis, instead express spider silk proteins in transgenic organisms, a strategy that offers significant advantages for both scaling and arachnophobes. But to generate those silks, spiders produce multiple silk proteins, each with distinct properties. According to the World Spider Catalog at the American Museum of Natural History, there are 44,540 known spider species. Some, like the widely studied *Nephila clavipes*, produce massive webs comprising at least five distinct silks—some stronger, some weaker, some elastic, some less so; two additional silks are used to protect *N. clavipes* offspring. Tarantulas spin no webs at all, instead putting out feeler lines to detect approaching prey. A recent genome sequencing analysis—referred to by the author as "silkomics"—identified 19 candidate silk genes in the African social velvet spider, *Stegodyphus mimosarum*, and 7 in the Brazilian white-knee tarantula, *Acanthoscurria geniculata*. [1]

"Spider silk proteins are actually a class of materials," says Thomas Scheibel, Chair of Biomaterials at the University of Bayreuth, Germany.

And many in the biomedical community see great promise in them.

A Day at the Museum

In 2009, the American Museum of Natural History in New York played host to an extraordinary 11-foot by 4-foot textile made of *N. clavipes* spider dragline silk—a magnificent fabric whose natural shimmering golden color reflects the spider's common name: the Golden Orb weaver.

That textile was the labor of four years and dozens of people—not to mention over one million wild spiders silked by hand in Madagascar. That's obviously wholly impractical for production work in the lab (or the textile industry). But that doesn't mean researchers can't use native spider silk on a smaller scale.

Jörn Kuhbier, a plastic surgeon and medical researcher in the Department of Plastic, Hand and Reconstructive Surgery at the Hannover Medical School in Germany, was a doctoral student in 2006 when he joined a lab that was exploring the biomedical application of spider silk. At the time, he wasn't exactly president of his local spider-appreciation club: "I did not like the idea of being in a room together with a spider," he says. Nevertheless, he was tasked with developing methods to grow cells on spider silk fibers, a first step towards using the material for tissue engineering.

The team's collection of *N. clavipes* spiders was housed in unused offices, where they were allowed to roam free and spin webs. Kuhbier and his colleagues created a map of each spider's approximate location in the office. The spiders were each named, and pins bearing a spider's name were affixed to the map to track which spiders were used and when, "to be sure that, for example, when Rosy was harvested on Wednesday, that she does not get harvested on Thursday as well."

Golden orb spiders are large—about the size of the palm of the hand—but they rarely bite and are relatively harmless to humans. To collect their silk, Kuhbier would nudge a spider from its web into a plastic box, immobilize it on a Styrofoam cube (without harming it), and gently pull the dangling dragline fiber from the animal's abdomen, winding it around a spindle. Kuhbier says he could pull up to 200 meters per animal in about 45 minutes, at which point the spider was returned to its web. A YouTube video on the Malagasy textile illustrates how delicate that process can

be: "You can just see these diaphanous spider silk strands kind of floating around the hands of these individuals," a museum curator explains. [2]

At first, Kuhbier did his silking manually; later, a motorized device was built in collaboration with the University of Hannover that extracted and wound the fiber more regularly and evenly—an idea that, as the team discovered after two

years of work, actually dates back to the eighteenth century, when a Spanish monk in Madagascar described perhaps the first ever spider silk-harvesting apparatus.

Collecting the silk was not the only hurdle though. "Spider silk has the ability to supercontract when it comes in contact with water." In aqueous solution—like, say, the culture media cells are grown in—the material knots up into an unusable bundle. Kuhbier needed a way to keep the silk taut.

It took him about a year and a half to develop a solution. Dentists, he recalled, often use an inexpensive bendable wire to hold dental prosthetics in place. Why not use that wire to create a "weaving frame" for spider silk? Such an assembly should prevent the silk from clumping, thereby retaining its structure when submerged in aqueous solutions.

The end result was a tiny golden silk tapestry 5 or 20 mm on a side—a stamp-sized golden miniature of the textile in New York. The structures are highly stable, he says—they can be washed in ethanol and autoclaved, and also seem to be resistant to both bacterial and fungal degradation. And they are small enough to fit in the wells of 6-well microtiter culture dishes. When Kuhbier seeded those frames with NIH 3T3 fibroblasts, he found that the cells not only adhered to the fibers (which are visible in photomicrographs due to their strong autofluorescence) but also proliferated, migrated, and thrived. [3] "They were in a healthy state and vital."

The frames also support true 3-D cell culture. In 2011, Kuhbier co-authored a study in which his weaving frames were used to build "artificial skin." [4] Here, the frame was stuffed with a ball (or "clew") of silk fibers to create a contiguous 3-D structure sandwiched between the two sides of the weaving frame. The silk frame was populated first with mouse embryonic fibroblasts and later with a surface layer of keratinocytes, which were grown at the liquid-air interface to force a more epidermal configuration. The resulting structure, he says, "formed skin-typical markers

A spider silk harvesting apparatus. Source: Simon Peers collection.

Randy Lewis in his lab, with a spool of silkworm silk. Photo by Jeffrey M. Perkel.

[visible] in immunofluorescence. So it seems to be a very good matrix for cell culturing.”

Wrangling Silk Genes

But that doesn’t mean the silk is convenient to use. Maintaining and silking live spiders is laborious and—Malagasy silk weavers notwithstanding—unscalable. As a result, many researchers concentrate on the silk genes themselves, mixing and matching protein segments to produce materials with the properties they desire.

As a graduate student, Anna Rising, now Associate Professor of Neurobiology at the Swedish University of Agriculture Sciences and the Karolinska Institute in Stockholm, decided to clone the gene encoding dragline silk protein from the nursery-web spider, *Euprosthenops australis*. Dragline silk forms the outer edges and structural support of a spider’s web; it is the silk spiders use to catch themselves when they fall. *E. australis* produces an exceptionally strong dragline, with a tensile strength of 1.5 GPa. But almost nothing was actually known about the organism itself, including where to find it, how to catch it, and whether or not the creature was poisonous.

Nevertheless, Rising traveled to South Africa to bag herself some spiders. Here’s the thing, though: It’s not that easy to find a spider in the wilderness. She and her guides first tried using headlamps to reflect the spiders’ eyes. But there are many animals in the bush, and many sets of eyes. Eventually, they realized their best bet was

to search for the animals’ distinctive funnel-shaped webs, which extend from a tree to the ground, where the spider waits in a burrow.

Even after locating the webs, Rising ran into trouble. “We struggled to catch them because they are so fast. They kept running down into this burrow, and then it was impossible to get hold of them.”

Eventually, the team found their solution: When the spider was in the web, a team member would shove a knife into the burrow, blocking the spider’s retreat, at which point “you could walk up to it and capture it in a jar. So, that was really easy, once you knew how to do it.”

In the end, Rising collected about 100 specimens, from which she and her team isolated the major ampullate gland that produces dragline silk. From there, they produced cDNA libraries and cloned parts of the spider’s major ampullate spidroin 1 gene, *MaSp1*.

Today, Rising has no spiders in her lab, save those that wander in from the outside. She sacrificed her specimens shortly after collecting them. *E. australis*, she says, are “very beautiful,” with “striped legs and a yellow abdomen and white head. I think they have eight eyes.” But, are they poisonous? “I didn’t know then and I still don’t know. I was never bitten, and nobody else [was], either.”

Not all silk is equal

Every spider silk protein is a little different, but most contain N- and C-terminal domains flanking as many as 100 alanine-

Anna Rising in South Africa. Photo courtesy of Anna Rising.

and glycine-rich repeats. *E. australis* *MaSp1* is about 10 kb long, but coding sequences can extend out to 15 kb or longer. The size and repetitive nature of the structure makes it difficult to manipulate and express intact silk proteins in transgenic organisms—bacteria will excise parts of the gene, for instance, and the proteins can aggregate—so researchers typically develop smaller custom protein designs.

Lewis says his lab has designed some 36 distinct silk protein variants ranging in size from 3 to 8 kb, each with unique material properties. Most of those are expressed in (and codon-optimized for) bacteria, but two are made in goat's milk, from which he can collect 15–20 g per week, and some are expressed in alfalfa (hay), which could yield nearly 220 kg per acre. Lewis also has expressed spider silk proteins in transgenic silkworms. Recombinant silk containing just 5% spider protein, he says, is already 50% to 70% stronger, and 40% more elastic, than silkworm silk alone.

Scheibel's lab has made more than 40 silk variants, all of which they produce in bacteria. His "workhorse," called eADF4(C16), is a 50 kD protein comprising 16 repeats (but neither terminal domain) of the *Araneus diadematus* ADF4 protein, an MaSp1 homolog.

Rising's variant, called 4RepCT, was developed under the guidance of principal investigator Jan Johansson, and comprises just the four C-terminal repeats of MaSp1 plus the C-terminal domain. "What the team did was to try to find the shortest protein that we could produce in *E. coli* and that, after purification, formed macroscopic fibers," Rising explains.

But being macroscopic does not make the fibers silk, exactly. 4RepCT, for instance, has only about 10% of the tensile strength and one-sixth the elasticity of natural silk.

Of course, biochemists also have different needs than spiders, so in the end it may not matter. Spiders need a fiber strong enough and elastic enough to catch an insect in flight. They need the material to be stable enough to be stored for extended periods as a liquid in their glands, and yet primed to form fibers quickly enough to catch them as they fall. And they have to be able to do all that at room temperature and pressure, more or less. Researchers only need something approximating the properties of the biological material they are studying, they can afford to wait hours for polymerization to occur, and they can prepare their materials under a variety of conditions.

Unlike spiders, researchers can also produce silk proteins in any form

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Silky Tissue

Nobody claims that every cell culture lab needs spider silk; its sweet spot seems to be tissue engineering. Spider silk can guide cell growth *in vivo*, produce a protective coating for implants, and serve as a matrix into which cells may be seeded *in vitro*, producing a material sturdy enough for implantation.

In general, says Wendy Bray, who manages the cell culture matrices portfolio at Thermo Fisher Scientific, researchers use three-dimensional cell culture matrices to mimic the cell's normal physiological niche. In the body, she explains, cells do not normally exist in two-dimensional sheets; they interact with other cells and receive a steady stream of signals from all sides. Some immortalized cell lines can grow without those cues, but studies have repeatedly documented differences in morphology, gene expression, and other behaviors from cells grown under more physiological conditions. Primary hepatocytes, for instance, will not grow well on standard culture dishes, Bray says; they prefer to be sandwiched between layers of collagen and murine extracellular matrix extract. "They need to be totally surrounded by that type of an environment in order to behave normally," she says.

There's no shortage of commercial materials providing some of those features, including scaffolds, hanging drops, and hydrogels. What spider silk brings to the table is biocompatibility, biodegradability, tunable mechanical stability, and, at least for recombinant protein, a defined chemical composition. "It's perfect for 3D scaffolding," says Fritz Vollrath, Professor of Zoology at Oxford University.

—J.P.

they want. Lewis spent \$100,000 on a custom-built apparatus that extrudes spider silk through a 5 micron needle—the resulting shear force converts the material from liquid to fiber—and then stretches and spins the material into something that looks like kitchen twine, yet is pure white and surprisingly soft. But he and others can also create foams, like the milk in a latte, transparent films, hydrogels, and other formats. "We have seen [that] pancreatic cell islets maintain their specific islet architecture and stay viable for longer times when cultured on a soft and porous material compared to a flat and rigid surface," says My Hedhammar, Associate Professor at the Swedish University of Agricultural Sciences and R&D Director of Spiber Technologies, a company she cofounded with Rising and others to commercialize spider silks.

The most common silks are blank canvases, says David Kaplan, Professor and Chair of Biomedical Engineering at Tufts University. "They have very little specific cell-activating chemistries, which means you can use them as a nice scaffolding onto which you can add selectively other things as you wish."

Kaplan, for instance, has modified silk proteins to nucleate glass and hydroxyapatite to make them stiffer. Hedhammar and Scheibel have independently coupled silk proteins to cell-adhesion moieties, such as the integrin-binding motif, RGD, to cause cells to bind the material more tightly. "If we stain the cells on spider silk compared to a [untreated] tissue culture plate, we can see that these adhesion sites are pronounced on the spider silk but not on the other. And that goes hand in hand with the proliferation—they grow better when they adhere in a natural way."

Presumably, says Lewis—who has cultured Chinese hamster ovary, fibroblast, and retinal cells on silk protein—the material could be modified to attract or repel specific cell types to accelerate, for instance, healing processes *in vivo*.

Significant testing remains to be done before any such benefits come

to pass. At the moment, researchers are still working out the basics of structure and function—why different silks have different properties. Should they unlock such secrets, and figure out how to exploit them, the bioprospectors could come a-running. Lewis, whose office in Utah features a "5 SILK" Wyoming vanity plate, has already staked his claim. But with nearly 45,000 documented spider species, there's still plenty of room for others.

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