Organ Weaving: Woven Threads and Sheets As a Step Towards a New Strategy for Artificial Organ Development[a]

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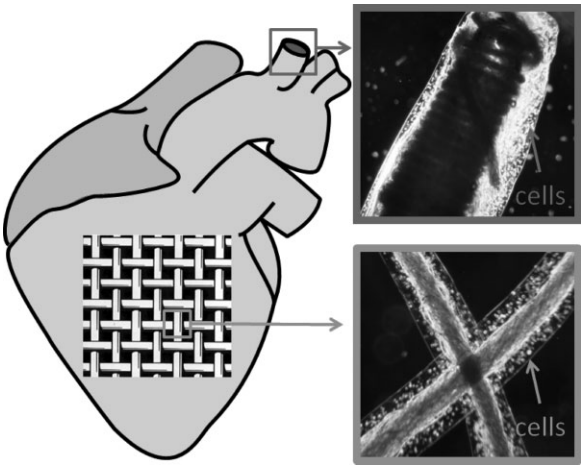
The concept of ''organ weaving'' is presented, a fabrication technique that can be an attractive option for the development of artificial tissues and organs. ''Living threads'' are created by immersing threads that are soaked in a $CaCl_2$ solution into a sodium-alginate-loaded cell suspension bath, encapsulating the cells and creating a bio-friendly, easily manageable starting material for building up larger scaffold structures. Such living threads have the advantage of being a particularly mild culturing medium for mammalian cells, protecting the cells during subsequent processing steps from dehydration and other rapid changes in the chemistry of the surrounding environment. Connecting different types of threads into 3D objects gives unique opportunities to address tissue engineering challenges.



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[a] **Supporting Information** for this article is available at Wiley Online Library or from the author.

Introduction

Tissue engineering, the multidisciplinary enterprise involving the development of biological substitutes that restore, maintain or improve tissue function or a whole organ,[1] is a highly diverse and evolving discipline. Most broadly, tissue engineering involves the creation of scaffolds to provide mechanical support and to host healthy living cells.

The different approaches to tissue engineering can be broadly categorized as either build-to-stock manufacturing techniques, or as rapid prototyping. In the former category, the production plans are based on a build-ahead strategy, where a stock of different sizes and dimensions of products is prepared ahead of time and stored until needed. One such approach involves the use of tissue scaffolds that are biological in origin, i.e., decellularized xenogeneic,

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allogeneic or cadaveric materials.[2–4] In such cases, an artificial tissue or organ is prepared from material taken from another organism, which has been decellularized by perfusion, and is then loaded with the appropriate cell culture(s) and cultivated under specific conditions to promote the desired proliferation. While this offers a direct route to a biomorphic system, it requires a stockpile of organs and tissues of a broad size range to fit to the recipient. Of course, there are also risks involved, such as infection and pathogen transmission, plus immunoreactivity/biocompatibility issues.[5]

The second broad category of manufacturing techniques, solid free-form fabrication, demonstrates a high degree of customization and free-form fabrication. These computer-assisted design/manufacturing (CAD/CAM)-based technologies involve first preparing a CAD file, outlining the dimensions of the prototype, which is then translated into a series of commands to build the system up. Stereolithography,[6,7] inkjet 3D printing,[8–10] cell patterning via biomaterial deposition[11] and extrusion[12,13] are examples of this approach, which permits a high degree of spatial resolution and control of the order of tens of micrometers. While these techniques have gained a lot of attention over the years in academic laboratories, a number of important unresolved technical issues remain in the field of soft tissue engineering rapid prototyping, including limitations in throughput and scalability.[11,14] The kinds of anchoring cell types of common interest in tissue engineering are only viable in suspension for roughly half an hour to an hour after trypsination; while these techniques are able to deliver with excellent spatial precision, the throughput is typically low ($<1\,mL \cdot h^{-1}$), meaning that the volume of tissue that can be cultivated is limited to structures with inherently high surface-to-volume ratios, which do not require vascularization to overcome diffusion limits.

Another subset of solid free-form fabrication involves fused deposition, which typically offers lower feature resolution, but significantly higher throughput. In such applications, cell suspensions and clusters are dispensed using CAD software to guide the deposition process.[14–16] This approach allows for larger structures to be prepared, though a significant amount of specialized equipment is needed, as well as a significant amount of computer programming.

Motivated by this need for better suited fabrication tools for tissue engineering, there was an interest in searching for alternative processing techniques that would allow us to create larger biomaterial structures, preferably with controlled 3D internal architecture, and preferably with the ability to incorporate living cells during the manufacture process. One such idea considered was whether the process of fiber weaving could be applied to tissue engineering. The idea was based on the observation that weaving technologies have been developed over the centuries to create structured materials with high degrees of complexity, and are routinely performed on a massive, industrial scale; the logic was that if the technology already existed to handle materials with good precision, it should be also applicable to living biomaterials. While numerous knitted fabric-based tissue scaffolds have been reported and even commercialized,[17–19] and the encapsulation of cells onto fibers has been applied to tissue engineering,[20] surprisingly, the combination of cell-loaded fibers woven or knitted into 3D architectures remains unreported, to the best of our knowledge, in the open literature.

Experimental Section

Materials

Sodium alginate (Cat. No. CAA W20,150-2), $CaCl_2$ (Cat. No. C1016), brilliant blue G (Cat. No. 27815), Hoechst-33342 (Cat. No. B2267), paraformaldehyde (Cat. No. 158127) and ethylenediaminetetraacetic acid trisodium salt hydrate (Cat. No. 106321) were all purchased from Sigma-Alrich (St. Louis, MO, USA). CellTracker Green CMFDA (Cat. No. C7025) was obtained from Invitrogen (Carlsbad, CA). PBS buffer (Cat. No. L1825) was purchased from Biochrom (Berlin, Germany) and L-glutamine (Cat. No. BE17-605E) from Cambrex (Ververs, Belgium). Inactivated fetal calf serum (Cat. No. A15-118), RPMI 1640 cell culture medium (Cat. No. E15-840) and penicillin/streptomycin (Cat. No. P11-010) were purchased from PAA (Pasching, Austria), trypsin/versene mixture (Cat. No. 17-161E) and Dulbecco's modified Eagle medium (DMEM, Cat. No. BE12-741F) from Lonza (Walkersville, MD) and surgical suturing "Polyglactin 910" (Cat. No. V492) from Ethicon (Norderstedt, Germany).

Red, white and black polyester threads were obtained from PaKo-Produktserice (Landsberg, Germany) and syringe needles (Sterica - 0.90×70 mm BL/LB, B/Brown; Cat. No. 722.1) were purchased from Carl Roth (Karlsruhe, Germany). Greiner® 50 mL poly(propylene) centrifuge tubes (Cat. No. T2318) were purchased from Sigma-Alrich (St. Louis, MO, USA).

All cells lines were kindly provided by Dr. Tobias Pöhlmann (siRNA Laboratory, IOMC Friedrich Schiller University, Jena, Germany).

Equipment

Cells counts were determined in a Neubauer hemocytometer chamber obtained from Blaubrand, Brand (Wertheim, Germany). Inverted light microscopy images were taken using an AXIOVERT 40 CFL microscope (Zeiss, Jena, Germany) using 10× and 2.5× objectives. Fluorescence images were taken using a Leica DMRXP microscope fitted with a 10× objective and a 0.25 FOV.

The Dropjet MJ-E-130 continuous printing system from Microdrop Technologies GmbH (Norderstedt, Germany) was used as a printing device. The Dropjet continuous printer was fitted with a 100 μm inner diameter nozzle, which was loaded with 10 mL of sodium alginate solution. The solution was processed using a back pressure of 200 to 380 kPa, where it was dispensed downwards into

Table 1. Summary of cell types used in this investigation.

Cell type	Medium	Concentration [10^6 cells · mL^{-1}]	Application	Additional treatment prior to threads preparation
L929	RPMI 1640	2	extrusion	1.17×10^{-3} M of brilliant blue G dye
L929	RPMI 1640	0.25	viability test	–
L929	RPMI 1640	1	weaving	–
L929	RPMI 1640	1	double layer prep.	CellTracker Green CMFDA
MCF-7	DMEM	1	double layer prep.	Hoechst-33342
HepG2	DMEM	1	weaving	–
HEK-293	DMEM	1	weaving	CellTracker Green CMFDA

a reservoir containing 50 mL of a 1.35 M solution of $CaCl_2$, which was sitting 5 cm below the nozzle.

Cell Culture

Mouse fibroblast (L929) cells, (passages 10–15) were grown in 5% CO_2 at 37 °C in tissue culture plastic flasks in RPMI 1640 growth medium, supplemented with heat inactivated fetal calf serum 10 vol%, penicillin (100 U · mL^{-1}) and streptomycin (100 mg · mL^{-1}). A summary of the cell culture experiments and their components can be found in Table 1.

Human breast cancer (MCF-7) cells, human liver cancer (HepG2) cells and human embryonic kidney (HEK-293) cells (passages 10–15) were grown in 5% CO_2 at 37 °C in tissue culture plastic flasks in DMEM supplemented with heat-inactivated fetal calf serum 10 vol%, penicillin (100 U · mL^{-1}) and streptomycin (100 mg · mL^{-1}). After the culture had achieved 75 to 85% cellular confluency, the cells were washed in the standard T-75 cell culture flask with 5 mL of PBS and trypsinized for 5 min with 0.75 mL of trypsin before mixing with sodium alginate.

Viability Testing

The cells within the gel scaffold were dyed using a combination of living/dead cell staining. First, the threads were placed in 12 well plates with 3 mL of cell culture media per well. Next, the cells were incubated at 37 °C. At every 48 h interval, three of the wells were stained for viability testing, while the remaining wells underwent a culture medium change. To change the culture medium, the supernatant liquid was removed and replaced with 3 mL aliquots of fresh medium.

For the wells that were tested for viability, a 1 mL aliquot of CellTracker Green CMFDA ($5 \cdot 10^{-6}$ μM in PBS) was added, and the cells were incubated with the dye for 15 min. Afterwards, the cells were centrifuged at 3 000 rpm for 5 min, and the supernatant was discarded. In order to suspend the cells (and to keep them separate for the purpose of accurate counting), an aliquot of 1 mL of a trypsin/versene mixture was added, and the cells were re-suspended. After 15 min of this treatment, the cells were centrifuged at 3 000 rpm for 5 min, and the supernatant liquid was discarded. For fixing the cells, a 2 mL aliquot of paraformaldehyde (4 mg · mL^{-1} in PBS) was added, and the cells were re-suspended and left for 30 min. The cells were centrifuged again at 3 000 rpm for 5 min, and a 5 mL aliquot of ethylenediaminetetraacetic acid trisodium salt hydrate (0.1 g · mL^{-1} in water) was added in order to remove any gel residues. The cells were re-suspended, and the suturing material was gently removed manually. The cells were centrifuged again at 3 000 rpm for 5 min, and 200 μL nuclei stain Hoechst-33342 (1 μg · mL^{-1} in PBS) was added. After 15 min, the cell suspension was studied by use of a Neubauer hemocytometer (Blau Brand) and a Leica DMRXP microscope fitted with a 10× objective. For detecting the living/(living + dead) cell ratios, the fluorescence signal was measured using a FITC filter (for counting the living cells) and without it (for counting all cells). This was repeated in triplicate every 48 h.

Weaving

The weaving process was carried out manually, yielding the woven structure illustrated in Figure 3 and 4. In the first step, four 70 mm long needles were immobilized in parallel with a distance of approximately 5 mm between each of them. Four previously prepared tubes containing HEK-293 cells were imposed on the syringe needles, one per needle. A total of 103 living threads containing L929 cells based on 20 cm long black polyester thread were prepared in the aforementioned fashion, as were 103 white polyester 20 cm long living threads, which were loaded with HEP-G2 cells. These threads were then placed one-by-one on the tubes in an alternating pattern of overlying and underlying threads on tubes. To be able to manipulate the threads between the tubes (putting, shifting, moving and tying at the edges) we utilized laboratory tweezers with sharp ends and syringe needles. The excess threads were cut out using scissors.

During the weaving, the structures were rinsed every 3 min with cell culture medium to prevent them from drying out. The structures were gently pushed out from the needles and subsequently incubated at 37 °C with 5% CO_2 in the incubator, immersed in DMEM cell culture medium. The structures as such were incubated for 24 h prior to microscope imaging.

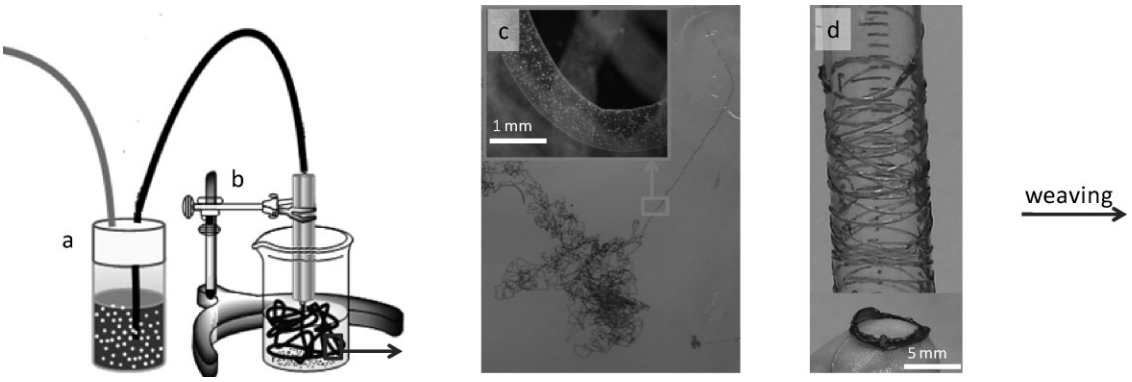

Figure 1. Making living threads, the basic building blocks of woven structures: (a) pressurized container with sodium alginate/cell culture; (b) linking via injection of alginate into an aqueous 0.180 M $CaCl_2$ solution, (c) living threads containing L929 mouse fibroblast cells, (d) living fibers wound up (see also Supporting Information).

Results and Discussion

In order to demonstrate a proof-of-concept for woven living materials, we prepared alginate hydrogel threads by means of a reactive extrusion technique (Figure 1),[18,19,21] and compared their weaving suitability to the threads developed via the reactive immersion method (Figure 2). Alginate is commonly known as biologically compatible material and is widely used for cell encapsulation applications,[22] as well as 3D scaffolding.[23–25]

Sodium alginate based living threads can be generated via simply pipetting an alginate solution into calcium chloride, or alternatively via injecting it from a syringe. However, with both procedures, the diameter of the threads is difficult to control. We have therefore made use of a continuous ink system, which is loaded with a sodium

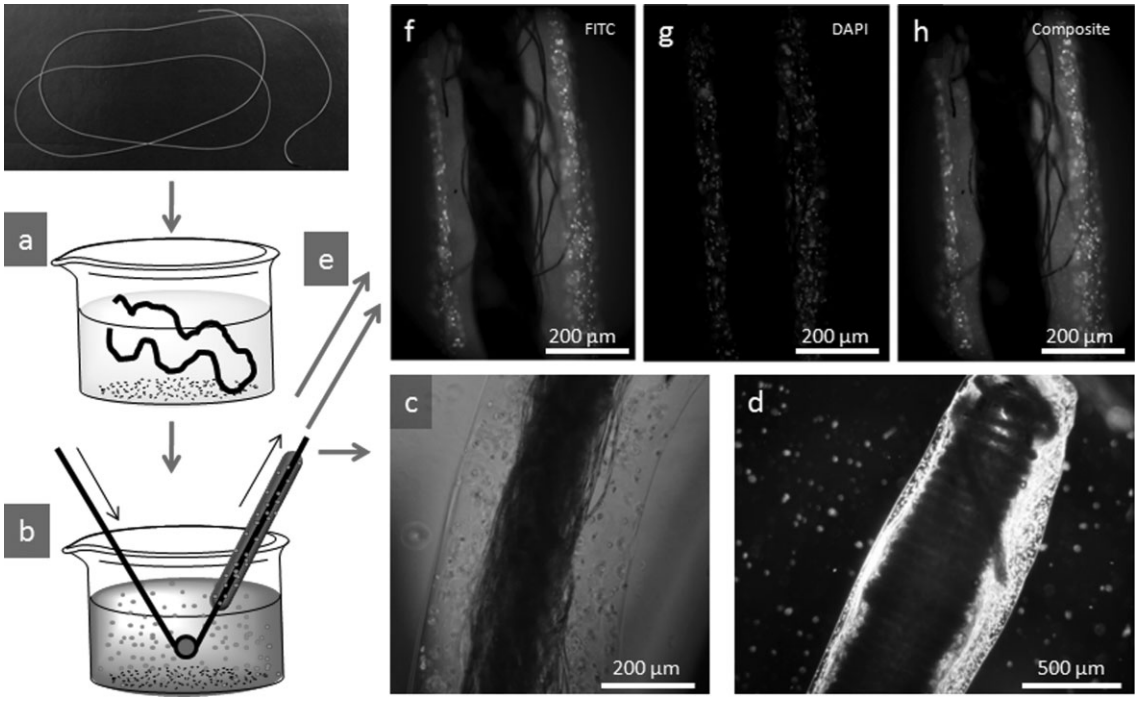

Figure 2. Fiber-supported living threads (improving mechanical properties and functionalization): (a) suturing threads are soaked in a $CaCl_2$ bath; (b) treated with sodium alginate cell suspension. (c) Single string coated with a cell-loaded layer of gel, and (d) the cell-loaded string was woven around a removable mandrel. (e) Complex multilayer architecture of thread can be obtained by subsequent repetition of step a and b. Fluorescence images of double-treated thread, including fluorescently labeled L929 cells stained with CellTracker Green CMFDA (f) and (g) MCF-7 cells stained with nuclei stain Hoechst-33342. (h), Overlay of images (f) and (g), showing two distinguishable layers of cells (see also Supporting Information).

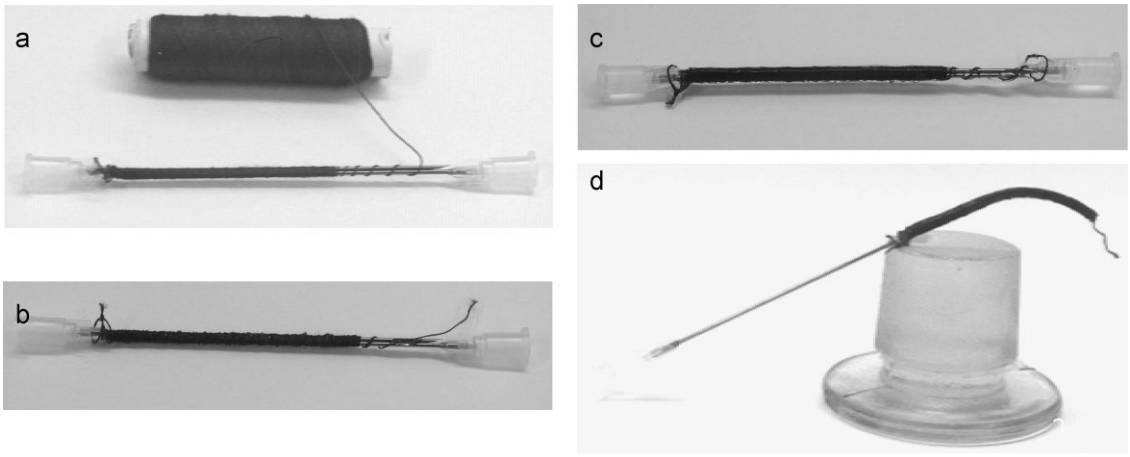

Figure 3. "Living tube" preparation: (a) A polyester thread was rolled over two parallel needles and (b) immersed into an aqueous 0.180 M $CaCl_2$ solution. The pipes were immersed into alginate solution (10 mg · mL^{-1} in DMEM) containing 10^6 cells · mL^{-1} of HEK-293 cells. (c) After removing the needles by shifting them in opposite direction, (d) the living tube is ready to use.

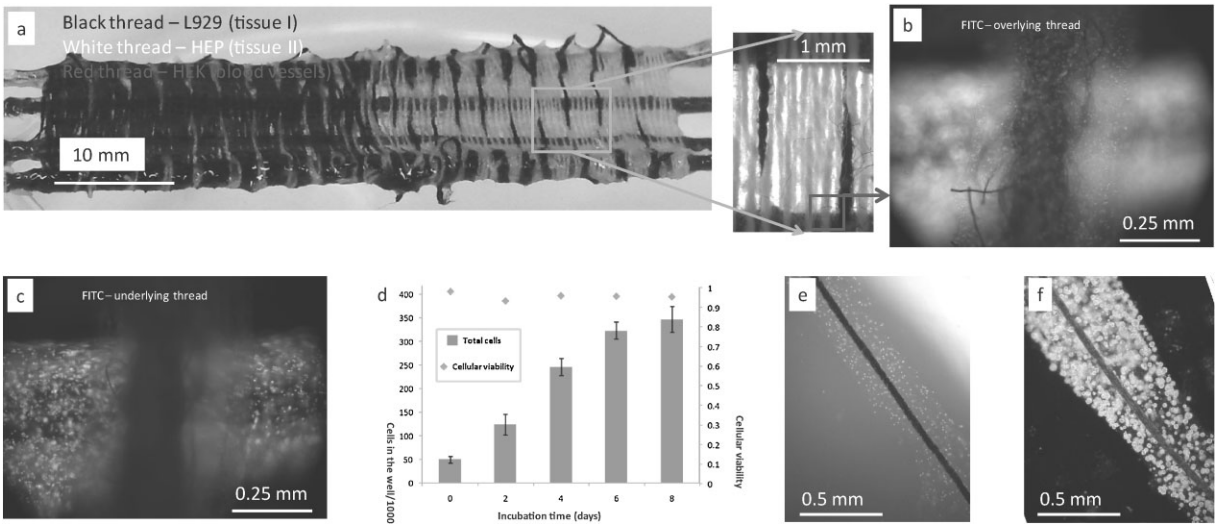

Figure 4. Organ weaving: connecting tissues using gradients. (a) An example of manual plain weaving using three cell lines. Magnified box: basic criss-cross pattern of plain weaving; (b) overlaid thread containing HepG2 cells; (c) underlying thread containing HEK-293 cells labeled (prior to weaving) with CellTracker Green CMFDA. Living threads were immobilized on polyester fibers. (d) Cell viability, as defined by number of living cells divided by the total number of cells, as measured by fluorescence microscopy. (e) Living thread immediately after preparation, (f) the same after 8 d incubation (see also Supporting Information).

alginate solution [10 mg · mL^{-1} in Roswell Park Memorial Institute (RPMI) 1640 cell culture medium], containing 2×10^6 living L929 cells per mL, and 1.17×10^{-3} M of brilliant blue G dye for improved visualization (see Figure 1). In the present approach, solutions of sodium alginate were allowed to react with solutions of dicationic salts (e.g., calcium chloride), where each metal cation complexes with two alginate chains to form a highly stable complex,[26,27] rapidly forming a crosslinked network under mild conditions. Normally, the intent of continuous inkjet printing is the generation of a stream of individual droplets; however, by adjusting the pressure (and consequently the flow rate) of the stream, along with the distance between the nozzle tip and the hardening bath, the stream could be hardened and reacted before Rayleigh jet break-up could occur, resulting in continuous structures.

As demonstrated in Figure 1, the strand of threads obtained by reactive extrusion was compatible with

winding as a processing technique. However, these fibers were not able to be processed by direct manual weaving. The most noteworthy challenge that made them incompatible with weaving was that the structures were rather fragile, and lacked the structural integrity to handle the weaving process. As a consequence, we tried to improve the stability as well as the durability of the living fibers by re-inforcing with other materials.

A second approach to living thread fabrication was the idea of a reactive immersion method. In order to increase the mechanical ruggedness of the threads, the gel containing living cells was anchored on a fiber scaffold (Figure 2). As an initial investigative fiber-scaffold system, we selected poly[(lactic acid)-*co*-(glycolic acid)]- (PLGA)-based surgical suturing as the core fiber material, which ensures biocompatibility and controllable degradation of the scaffold. For the generation of larger structures, we used the more economically viable alternative of textile-grade polyester thread. In this approach, surgical-grade suturing material was soaked in an aqueous 0.180 M $CaCl_2$ solution, wrung out to remove excess liquid, and then passed through a bath containing a cell suspension loaded prepared from a sodium alginate solution (10 mg · mL^{-1}) in RPMI 1640 cell culture medium (See Figure S1 in the Supporting Information). By passing this thread through a second calcium bath, followed by a second sodium alginate/cell bath, a successive ablumenal coating of a difference cell type could be deposited. To illustrate this concept, two differently fluorescently labeled cell types were deposited in this manner. First, around the fiber core, a lumenal layer of fluorescently labeled MCF-7 cells stained with nuclei stain Hoechst-33342, was deposited, followed by a second, ablumenal layer of fluorescently labeled L929 cells stained using CellTracker Green CMFDA. When imaged using filtered fluorescence microscopy, the resulting images of the structure showed dramatically the multilayered internal structure of the thread [Figure 2(f)-2(h)].

Once the cell-loaded threads had been prepared, we investigated their use in constructing larger, three-dimensional and multilayered structures as models for blood vessels. The simplest applications for this technique involved wrapping the living thread around a removable mandrel [Figure 2(d)], but require additional crosslinking to afford an integral, cohesive structure. An alternative approach to this was to first soak the core fiber in $CaCl_2$, then wrap the thread around a mandrel, and then soak the entire structure in a cell/alginate bath (Figure 3). Additional layers could be added afterwards in an analogous fashion. In order to demonstrate the structural integrity of the resulting tubing, a 15 cm length of living tubing (2 mm diameter) was mounted to the outlet of the syringe and 10 mL of cell culture medium was pumped through it in 15 s while the pipe was resting on the surface of a Petri dish and the other end of the tube was placed in a pre-weighed centrifuge tube. The amount of liquid collected in the receiver was 9.8 g (SD = 0.1 g, $n = 4$), showing that the tubes were leak-free. However, occasionally some diffusion of liquid out of the tube was observed. The strategy for connecting different types of tissue is based on weaving two or more types of fibers containing the desired types of living cells (Figure 4). In terms of measured viability, the cells seem to tolerate the encapsulation process well, showing viability near unity at every measured time point [Figure 4(d)]. Vascularizable structures could be attained using strings that possess either a hollow or a quickly degradable core. 3D weaving can be used to create structures with a greater degree of complexity.[28] Additional functional components can later be threaded analogously through the matrix, as well. The mechanical endurance of "living" threads and woven sheets depends on the materials used as fiber reinforcements. In our case, we used different polyester fibers, and, in each case, the strength of the fibers was greater than 500 g (measured by mounting and hanging a 500 g weight to a single length of each thread). The enforced threads were successfully used for manual weaving of the sheets that were later found to be stable and compact during culturing conditions. Altogether, these results show that threads and woven sheets withstand handling and biomechanical testing. Finally, the mechanical endurance of the woven structure will depend mainly on the interaction between the cells and external cellular matrix when applying degradable surgical suturing as enforcing material for the gels.

While cell viability techniques have been reported that can facilitate in-situ measurements on 3D scaffolds,[29] viability tests were difficult to carry out on intact structures at the current stage, due to the fact that the cells were not always readily visible in larger 3D objects. Instead, we reversed the gel state of the alginate that formed the polymer matrix by addition of a dilute solution of the trisodium salt of ethylenediaminetetraacetic acid (EDTA), similar to matrix digestion of 3D scaffolds reported elsewhere,[30] and then quantified the cells using a standard dual fluorescent dye approach. To distinguish living from non-living cells, all cells were stained, both living and dead, with Hoechst-33342, to give a measurable signal in the DAPI fluorescence channel (351 → 461 nm),[31] and afterwards, all living cells were stained selectively with CellTracker Green CMFDA, to provide a measurable signal using the fluorescein channel (494 → 521 nm).[32,33]

Extending the idea of a fiber re-enforced multilayered alginate structure to larger structures, one particularly intriguing idea was to build cell encapsulating systems that were both hollow as well as fiber re-enforced. Such materials could be useful for artificial blood vessels. In this approach, cotton gauze was wrapped around a poly(propylene) mandrel, soaked in a $CaCl_2$ hardening bath, wrung

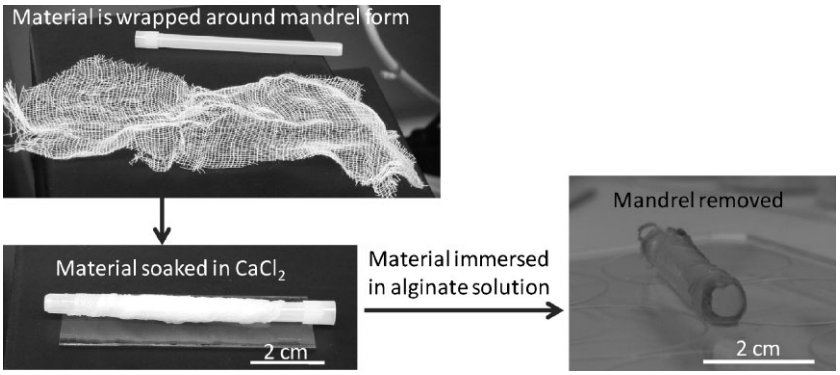

Figure 5. Preparation of living pipes. From top to bottom: the solid components prior to assembly, the wrapped textile is soaked in a hardening bath solution, soaking in alginate cell solution and finally removing the mandrel yields a free-standing living tube.

out to remove excess solution and then immersed in a crosslinkable alginate solution (Figure 5). The mandrel was removed to yield a hydrogel structure seeded with living cells, which may be used in the near future for artificial blood vessels.

Conclusion

In conclusion, we have presented the proof-of-concept for a new type of artificial organ development. Living structures with 3D architecture could be obtained in a weaving process ("organ weaving") using living cell-encapsulating fibers. This work is an attempt to set out our vision for a direction we would like to see pursued in the field of tissue engineering, where "organ weaving" is meant as an approach by which living cells, matrix materials, growth factors and other components are built into rugged, simple building blocks that can be manipulated into larger target 3D structures using facile, well-established techniques.

A method for fabricating hydrogel strings was demonstrated using reactive extrusion and immersing methods. Living cells could be successfully encapsulated into hydrogel strings. The fibers were then used to construct living threads and woven sheets. The methods outlined could in the future be used for the construction of complex structures, such as artificial blood vessels, prosthetic materials for reconstructive surgery and artificial cartilage; with tissue components available, larger, more complex architectures, such as organs, are envisioned as a future end goal.

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