3D-printed synthetic tissues

Michael J. Booth and Hagan Bayley

Department of Chemistry
University of Oxford
12 Mansfield Road
Oxford, OX1 3TA, UK
Abstract

"Bottom-up" approaches in synthetic biology have been used to construct synthetic cells from simple biological components. By contrast, relatively little work has been done on synthetic tissues in which collections of cells cooperate to achieve functionality that cannot be generated by individual compartments. We have developed a 3D printer, which can create structures containing hundreds or thousands of communicating aqueous droplets arranged in programmed patterns. These tissue-like materials can adopt properties such as the ability to fold or conduct electrical signals. Further, the properties of the materials can be extended, so that they become true synthetic tissues through the performance of sophisticated functions such as protein synthesis. In addition, we have shown that 3D-printed synthetic tissues can be controlled and energized externally, for example by light. Printed synthetic tissues might find a variety of uses in medicine and even be interfaced directly with living tissues. Because they contain no genome and cannot replicate, synthetic tissues are comparatively safe for medical applications.
3D printed aqueous droplets
We have developed a 3D printer to create structures containing hundreds or thousands
of aqueous droplets arranged according to programmed patterns. Recently, we have
used the approach to produce synthetic tissues, which ultimately may have applications
in medicine, for example in the repair of damaged organs. By tissues, we mean biological
materials with emergent functional properties that surpass the sum of the capabilities of
the individual compartments. While bottom-up synthetic biology proceeds apace,
including the construction of synthetic cells, there has been little work on synthetic
tissues in which these cells behave in a cooperative manner.

Curiously, our approach to synthetic tissues evolved from an attempt to miniaturize
single-channel current recording through biological channels and pores in lipid
bilayers. We form two aqueous droplets (~200 nL) in an oil that contains lipid
molecules. A monolayer of lipid forms around each droplet, and when the droplets are
brought together a bilayer is formed at the interface, which we have named a droplet
interface bilayer (DIB). With a membrane protein of interest inserted into
the bilayer and an electrode in each droplet, current recordings are made. DIBs have
been widely adopted for biophysical studies.

If two droplets can be joined together, why not three, four or ten? We proceeded to build
multi-droplet systems, first in two and then in three dimensions, in which each droplet
is connected to one or more of its neighbours through a DIB. For example a 3-layer, 10-
droplet pyramid was made by the micromanipulation of droplets containing
paramagnetic beads. These networks can be endowed with useful
functional properties. For example a 4-droplet network, containing a diode-like mutant
of the α-hemolysin (αHL) transmembrane protein pore arranged vectorially in each DIB,
carries out full-wave rectification of an electrical signal (Figure 1c).

The methods we had devised to build small droplet networks were not practicable for
larger structures. For this, we constructed a piezo-based 3D printer capable of printing
thousand of aqueous droplets (~50 pL) in patterned 3D assemblies. These
printed structures can be viewed as mechanically resilient systems, resembling soft
balls held together by springs. The printed droplet networks can be formed inside oil
drops in an aqueous environment, in which case the final structure is encased in a lipid
bilayer through which communication with the external environment can be mediated
by membrane proteins. Again, these structures can afford functional properties. For
example, a 4-petal structure was printed onto a flat surface from layers of droplets with
different internal osmolarities. After printing, water flow across the bilayers caused the
structure to fold into a hollow sphere. With their multiple communicating
compartments and properties analogous to living organisms (here shape change), we
consider these printed structures to be "tissue-like materials".

Controlling synthetic tissues with light
Our early work used only simple reagents inside 3D-printed droplets to produce
electrical communication and shape change. Furthermore, the properties of the
materials could not be altered after printing. To produce synthetic tissues, namely
materials closer to biological tissues, we needed to enhance the properties of the
constituent compartments so that they more closely resembled living cells. Therefore, we elaborated the droplets by adding the ability to synthesise protein from encapsulated DNA with an in vitro expression system. Such synthetic cells had previously been made from individual vesicles bounded by lipid bilayers, but these entities had not been elaborated into multi-compartment systems. By printing networks of hundreds of synthetic cells, we sought to produce the emergent properties associated with tissues by allowing communication between the compartments, which had been a principal feature of our simple tissue-like materials. Additionally, we aimed to control the synthetic tissues externally, by using an in vitro expression system turned on by light.

We quickly found that our original aqueous droplet networks were only stable when they contained simple salt solutions with at most very low concentrations of protein. The lipid bilayers between each compartment broke when a high concentration of protein was present, such as that in an expression system, which contains ribosomes and many different enzymes. We overcame this by altering the lipid composition of the DIBs to include lipids that contained head-groups derivatized with polyethylene glycol (PEG), which projected into the aqueous interiors of the droplets. PEG-head-groups have been shown previously to stabilize bilayers, by shielding them from the adjacent solution. Aided by the PEG lipids, we printed synthetic tissues comprising hundreds of synthetic cells patterned in defined geometries. Protein could then be expressed inside each of the synthetic cells.

We next sought to externally control the activity of these synthetic tissues. A common means by which to control biological systems is with light, which can penetrate living cells without disrupting membrane-bound compartments or upsetting cellular pathways. Two previously published methods have demonstrated control of in vitro protein expression with light. However, we developed our own way of controlling expression (Figure 3a). In particular, we required an off-state that was not leaky. This was accomplished by covalently attaching the small molecule biotin at several sites on the promoter region of the gene of interest. Biotin binds strongly to the large protein streptavidin, which was used as a steric blocker of the promoter sequence thereby preventing transcription of the DNA by RNA polymerase. Therefore, the first step in protein expression, the formation of messenger RNA did not occur. We also inserted photocleavable linkers between the promoter and the biotins, which allowed removal of the block with low energy ultraviolet light, permitting transcription followed by translation of the messenger RNA into protein. By optimising the number of biotins and their sites of attachment, we developed a tightly regulated light-activated DNA (LA-DNA) promoter.

By combining the stabilized aqueous droplet networks with the LA-DNA, we produced light-activated synthetic tissues. In one example, the αHL pore was produced within the synthetic cells by using the expression system under control of the LA-DNA promoter (Figure 3b). Following 3D printing, the synthetic tissues showed no functional activity, as the LA-DNA was not transcribed and no protein was produced. After irradiation, αHL was synthesised, forming pores in the DIBs through which electrical current could pass. By patterning the synthetic cells that contained LA-DNA during printing, or by using...
guided irradiation, extended 3D pathways of cells producing αHL were generated within synthetic tissues. This resulted in light-activated directional electrical communication realized with external electrodes (Figure 3c). Rapid, directional electrical signaling is precisely the role of neurons, although they work through a different mechanism. Therefore, our approach demonstrates the power of external light-control of expression and has parallels with the field of optogenetics, where neural activity is controlled by light in living organisms16.

We are presently expanding the repertoire of proteins that can be expressed inside the synthetic tissues. For example, light-activated channels and pumps generate currents across bilayers in the absence of electrodes. Synthetic tissues containing them could be used to transduce light signals or as micrometer-sized power generators. Patterning by 3D printing and by light will add valuable levels of complexity to these systems.

**Future directions**

A long-term goal is to use 3D-printed synthetic tissues in medicine. Potential uses include drug delivery and tissue replacement in surgery. In drug delivery, the multi-compartment nature of the materials will allow the release of binary or ternary agents in which components (e.g. an enzyme and a substrate) are combined at a desired site to generate potent effectors10. In surgery, synthetic tissues might be used to replace damaged tissue: for example, to provide a provisional electrical connection after nerve damage. Synthetic tissues are likely to be less problematic than therapies based on living cells, for which questions of immunogenicity and uncontrolled proliferation arise.

While the rapid progress in this new area suggests that these ideas are not far fetched, there remains a long way to go towards these and other applications. In the meantime, numerous issues of significant technical interest must be addressed. First, improvements in construction techniques and materials are required. Safety considerations suggest that synthetic tissues would preferably be robust rather than self-renewing. Hydrogel blocks, which are not restricted to a spherical shape, have been used as components for network construction in place of aqueous droplets17 (Figure 4a). Droplet networks might also be gelled or encapsulated after printing to provide robustness. 4D printing (printing followed by folding) might also be a useful construction approach, as our shape change experiments already suggest1. Larger objects could be made more rapidly through the use of 3D printers with multiple printing heads and printed objects themselves might be used as building blocks for extended structures.

Second, the synthetic tissues must encompass a far wider variety of functional properties. We have already explored shape change, electrical signaling and drug release1, 10. Additional possibilities include the in situ synthesis of short-lived biological effectors that can't be packaged into pills and drips (e.g. S-nitroso compounds or hydroperoxides), the ability to sense the environment and respond, the means to take up, modify and release molecules, the capacity to interact directly with neighbouring biological tissues and the ability to be energized from external sources (e.g. light). We have previously engineered derivatives of the αHL pore that mimic gap junctions18.
(Figure 4b), proteins that span two lipid bilayers and which could be used to connect synthetic tissues with biological tissues directly.

Third, additional means of external control of synthetic tissues must be devised and present approaches optimized. We have explored photocontrol (as emphasized here)\(^2\) and electrical manipulation\(^1\)\(^,\)\(^9\). Additional possibilities include magnetic\(^17\) and mechanical stimulation\(^19\). While, the ability to turn systems 'on' can be extremely useful, 'on-off' switches are obviously more desirable.

Still further in the future, we foresee a myriad of potential technologies, many of which are likely to be controversial, at least initially. We envisage autonomous soft robots\(^20\) based on 3D-printed synthetic tissues. We see printers capable of climbing into and printing directly in surgical sites and implants that allow humans to detect previously imperceptible molecules, sights and sounds.

**Author Biographies**

Michael Booth is a Junior Research Fellow at Merton College, Oxford. He is based at the University of Oxford in the laboratory of Professor Hagan Bayley. His research focuses on the assembly and applications of controllable synthetic cells and tissues. email: michael.booth@chem.ox.ac.uk

Hagan Bayley is the Professor of Chemical Biology at the University of Oxford. Major interests of his laboratory are the development of engineered pores for stochastic sensing, the study of covalent chemistry at the single molecule level, ultrarapid nucleic acid and protein sequencing and the synthetic biology of minimal tissues. email: hagan.bayley@chem.ox.ac.uk
Figure 1.
Droplet networks connected with droplet interface bilayers. (a) Two aqueous droplets, in an oil containing lipid molecules, spontaneously form lipid monolayers on their surfaces. When the two droplets are brought together a droplet interface bilayer (DIB) forms between them. (b) Side and top view of a 3-layer, 10-droplet pyramid. Scale bars, 200 μm. (c) Electrodes were inserted into a 4-droplet network, containing a diode-like mutant of the α-hemolysin (αHL) pore. The network rectifies an electrical signal.
Figure 2.
A 3D droplet printer. (a) The 3D droplet printer consists of glass capillary printing nozzles, each connected to a chamber enclosing a piezoelectric disk, which allows the ejection of picoliter aqueous droplets into an oil bath containing lipid. Here, two print nozzles produce a patterned 3D network. (b) A 4-petal structure, consisting of layers of droplets with different internal osmolarities, was printed onto a flat surface. After printing, water flowed across the bilayers causing the structure to fold into a hollow sphere. Scale bar, 200 μm.
Figure 3.
Light-activated synthetic tissues. (a) The LA-DNA carries the small molecule biotin attached through a photocleavable linker (PCL) at several sites on the promoter region of a gene of interest. Biotin binds strongly to the large protein streptavidin, which blocks transcription of the gene by RNA polymerase. Low energy ultraviolet light cleaves the PCL groups, removing the steric block. The DNA can then be transcribed into messenger RNA and translated to protein. (b) A light-activated gene, encoding a protein pore, is expressed inside synthetic cells, by using an in vitro expression system. The resulting protein pores form holes in the lipid membrane between the synthetic cells, allowing electrical communication. (c) Synthetic cells can be printed into patterned synthetic tissues, which exhibit directional signaling through defined pathways, shown here by the expression of a fluorescent protein.
Figure 4.
Advancing printing technology. (a) Hydrogel blocks can form networks with bilayers at each interface\textsuperscript{17}. Here, seven blocks with different shapes are connected by six interface bilayers. (b) Schematic and an electron micrograph of an engineered derivative of the $\alpha$HL pore that spans two lipid bilayers\textsuperscript{18}. The pore might be used to directly connect synthetic tissues with biological tissues. Scale bars, 20 nm.
References

1. Villar, G., Graham, A.D. & Bayley, H. A tissue-like printed material. *Science* **340**, 48-52 (2013).
2. Booth, M.J., Schild, V.R., Graham, A.D., Olof, S.N. & Bayley, H. Light-activated communication in synthetic tissues. *Sci. Adv.* **2**, e1600056 (2016).
3. Woolfson, D.N. & Bromley, E.H.C. Synthetic biology: a bit of rebranding, or something new and inspiring? *Biochemist* **33**, 19-25 (2011).
4. Holden, M.A., Needham, D. & Bayley, H. Functional bionetworks from nanoliter water droplets. *J. Am. Chem. Soc.* **129**, 8650-5 (2007).
5. Bayley, H. et al. Droplet interface bilayers. *Mol. Biosyst.* **4**, 1191-208 (2008).
6. Weatherill, E.E. & Wallace, M.I. Combining single-molecule imaging and single-channel electrophysiology. *J. Mol. Biol.* **427**, 146-57 (2015).
7. Schmidt, J. Membrane platforms for biological nanopore sensing and sequencing. *Curr. Opin. Biotechnol.* **39**, 17-27 (2016).
8. Wauer, T. et al. Construction and manipulation of functional three-dimensional droplet networks. *ACS Nano* **8**, 771-9 (2014).
9. Maglia, G. et al. Droplet networks with incorporated protein diodes show collective properties. *Nat. Nanotechnol.* **4**, 437-40 (2009).
10. Villar, G., Heron, A.J. & Bayley, H. Formation of droplet networks that function in aqueous environments. *Nat. Nanotechnol.* **6**, 803-8 (2011).
11. Noireaux, V. & Libchaber, A. A vesicle bioreactor as a step toward an artificial cell assembly. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 17669-74 (2004).
12. Allen, T.M. & Cullis, P.R. Liposomal drug delivery systems: from concept to clinical applications. *Adv. Drug. Deliv. Rev.* **65**, 36-48 (2013).
13. Gorostiza, P. & Isacoff, E.Y. Optical switches for remote and noninvasive control of cell signaling. *Science* **322**, 395-9 (2008).
14. Liu, M., Asanuma, H. & Komiyama, M. Azobenzene-tethered T7 promoter for efficient photoregulation of transcription. *J. Am. Chem. Soc.* **128**, 1009-15 (2006).
15. Estevez-Torres, A. et al. Sequence-independent and reversible photocontrol of transcription/expression systems using a photosensitive nucleic acid binder. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 12219-23 (2009).
16. Fenno, L., Yizhar, O. & Deisseroth, K. The development and application of optogenetics. *Annu. Rev. Neurosci.* **34**, 389-412 (2011).
17. Sapra, K.T. & Bayley, H. Lipid-coated hydrogel shapes as components of electrical circuits and mechanical devices. *Sci. Rep.* **2**, 848 (2012).
18. Mantri, S., Sapra, K.T., Cheley, S., Sharp, T.H. & Bayley, H. An engineered dimeric protein pore that spans adjacent lipid bilayers. *Nat. Commun.* **4**, 1725 (2013).
19. Sarles, S.A., Madden, J.D.W. & Leo, D.J. Hair cell inspired mechanotransduction with a gel-supported, artificial lipid membrane. *Soft Matter* **7**, 4644-4653 (2011).
20. Rus, D. & Tolley, M.T. Design, fabrication and control of soft robots. *Nature* **521**, 467-75 (2015).