Formation of polarised, functional artificial cells from compartmentalised droplet networks and nanomaterials, using one-step, dual-material 3D-printed microfluidics

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Abstract:
The bottom-up construction of synthetic cells with user-defined chemical organisation, holds considerable promise in the creation of next generation, smart, bio-inspired materials. Complex emulsions, droplet networks and nested vesicle structures, all represent candidate platforms for the engineering of segregated chemistries with controlled communication, analogous to biological cells. The microfluidic manufacture of such droplet-based materials, typically results in radial, or axisymmetric structures. In contrast, biological cells frequently display chemical polarity, or gradients, which enable the determination of directionality, and inform higher-order interactions in cellular communities or tissues. Here, we develop a dual-material, 3D-printing methodology, to produce microfluidic architectures that enable the construction of functional, asymmetric, hierarchical, emulsion-based artificial cellular chassis. These incorporate droplet networks, lipid membranes and nanoparticle components. The three-dimensional microfluidic channel arrangements enable symmetry-breaking, which facilitates the spatial patterning of droplet hierarchies. Such hierarchies are shown to produce internal gradients and hemispherically patterned and layered shells, alongside chemical compartmentalisation. Using this approach, we incorporate organic and inorganic components, including biologically compatible lipid-bilayers, within the same entity. In this way, we create functional polarisation that imparts individual and collective directionality, on the resulting artificial cellular structures. Such properties will increase the palette of functionality in chemically organised synthetic cells. This method enables the exploitation of polarity and asymmetry, in conjunction with compartmentalised and networked chemistry, for both single, and higher-order, organised structures.

Introduction:
The creation of artificial cells with functional properties, that are analogous to their biological counterparts, is envisaged to give rise to a wealth of opportunities, in a diversity of application areas, in both biotechnology and therapeutics [1-5], for example, in tumour cell targeted drug delivery [6], and artificial kidneys for clinical treatments [7]. Imparting functionality to artificial cells, is typically underpinned by organisation of chemical reactions, with the aim to engineer specific dynamic or responsive behaviours. This itself, is enabled by the spatio-temporal organisation of chemical reactions, and their selective, controlled communication [8-12]. The aspiration to engineer such chemical models which harness biology, represents a conceptual shift from linear systems with fixed physical-chemical properties, towards the
exploitation of emergent, non-equilibrium, and dynamic properties [13]. Such approaches are both inspired by, and become increasingly realisable with, our understanding of biological complexity, and the cell’s exploitation of emergent behaviours; for instance, those arising from (i) molecular self-assembly, (ii) the barrier properties of membranes, (iii) the compartmentalisation of multiple chemistries, and (iv) the process of symmetry-breaking, to develop new properties [14-22]. In this regard, and of particular interest, is the ability to manufacture miniaturised, asymmetric entities with spatially organised chemistries to dictate functional polarity [23]. Such features have been demonstrated to possess emergent and collective properties in solid-state materials [24, 25]. In biological systems, these properties are essential for dictating directional motility [26], governing cellular migration [27], enabling response to external chemical gradients [28] and defining directional growth [29, 30]. Consequently, there has been significant interest in incorporating such polarisable properties in artificial cells [31-38]. However, to date, the practical establishment of this has remained limited [39, 40].

Despite the importance of discrete chemical organisation in artificial cells, the majority of research in bottom-up synthetic biology, has focussed on the development of much simpler models. There has been significant interest in the construction of spontaneous, self-assembled, artificial cells, in the form of vesicles, colloidosomes, or phase separated oil-water, or water-water (coacervate) systems [41-45]. The exploitation of such processes can shed important light on the emergence of early life, and resultant structures may represent minimally complex, artificial cell chassis, often termed ‘protocells’. However, using these spontaneous self-assembly processes alone, it is challenging to recapitulate the controlled sub-compartmentalisation and spatial organisation of biological cells, and, therefore, the higher functionality of even simple biological systems. In this regard, it is notable that few biological structures are formed de-novo, instead being spatially and temporally constructed, or formed, from existing structures [46]. Consequently, droplet microfluidics represents and interesting parallel, where channel architecture and flow provide a route for directing the assembly of compartmentalised, and spatially patterned, chemically organised, soft-matter materials [22, 47-49].

Beyond these self-assembled systems, increased structural complexity has been sought in droplet networks [50-52] and compartmentalised vesicles [53], to produce interconnected, membrane-segregated compartments, as artificial cellular systems. Such materials have been constructed manually [18], by the printing or assembly of individual droplet units [20] and by using microfluidics [54] and demonstrated as networked reactors possessing emergent properties [55, 56]. Microfluidic construction of such materials offers the opportunity of scalable production, using massively parallel microfluidics [57, 58], and typically involves their formation from hierarchical water/oil emulsions. Ourselves, and others, have demonstrated the creation of droplet networks, segregated by lipid bilayers using microfluidic approaches [54, 59]. By extending this concept to a third phase and forming a triple emulsion, such droplet structures can be effectively encapsulated within a permeable, free-standing, hydrogel shell. This droplet assembly is able to communicate with its external environment, and be functionally enhanced by the integration of transmembrane proteins [59].

It is notable that the microfluidic formation of such hierarchical droplets, or higher-order emulsions, requires the integration of both hydrophilic and hydrophobic surfaces within the same microfluidic device. This determines the channel wetting properties and governs whether water or oil droplets form at a given junction. Consequently, the requirement to alternate between aqueous and non-aqueous wetting phases represents a fabrication challenge
to achieve the integration of both hydrophilic and hydrophobic channels. This may entail the complex assembly of multi-material, or surface functionalised, fluidic channels. Furthermore, traditional planar fabrication tools usually constrain droplet flows to a single plane, which creates 2D axisymmetric morphologies in double and triple emulsions. Consequently, in both lipid bilayer stabilised, and more traditional multiphase emulsions, a number of constraints are placed on the ability to create non-symmetric droplet structures in a well-controlled manner.

In this regard, asymmetric droplets, or so-called Janus droplets, have been explored as a means to break the morphological symmetry in droplets [60-62]. Janus droplets were first reported by Nisisako et al with microfluidic formation of such structures deterministically created in single emulsion laminar flows [63]. Janus particles have been used in applications that exploit the particle anisotropy, ranging from chemical sensors [64] to electrically activated, reconfigurable displays [63]. Double emulsions have also been used as a template to create Janus polymer structures, with the immiscible oil and water volumes templating the synthesis of two conjoined particle hemispheres [65]. Double emulsions with a Janus shell have been also been reported [66,67], but the Janus patterning has remained limited to a single phase, and in a single plane. The ability to expand such approaches and combine with droplet interface bilayer networks, could provide a means to pattern individual layers of hierarchical, membrane-based, cellular structures. Further, the ability to create droplet structures with symmetry-breaking in more than one plane, would enable the discrete patterning of each phase, and its orientation, in the droplet hierarchy. As a result, this could produce chemical gradients or polarised phases, and position compartments spatially within the fluidic or soft matrices. This would, therefore, facilitate the construction of synthetic cell chassis, with compartmentalised chemistries, access to membrane biochemistry, with overall spatial control of assembly. Such order would enable control over chemical polarity, and increase the functional complexity of artificial cells.

To realise this, we report here, the development of one-step, multi-material microfluidic devices with three-dimensionally arranged channels, to produce hierarchical droplets with discrete patterning of component liquid phases. Using this approach, we demonstrate the production of symmetry-breaking droplet structures, incorporating patterned organic and inorganic components, with biological lipid-bilayers for the first time. This increases the palette of functionality and afforded assembly control, in such chemically organised synthetic cells. We demonstrate the ability to impart polarisation driven behaviours in the constructs, by creating magnetically polarised, encapsulated droplet networks, that can orientate, rotate and migrate, as well as collectively establish a common orientation, and undergo shared collective motions in larger populations.

**Results:**

**Single-step 3D printed microfluidic devices for the production of multiphase emulsions:**

The advent of 3D printing has enabled the construction of three-dimensional fluidic architectures [68, 69]. In droplet microfluidics, hydrophobic fluidic channels govern the formation of water droplets in a continuous oil flow, and hydrophilic fluidic channels enable the dispersion of oil droplets in continuous water flows. Here, we developed a monolithically constructed, 3D fluidic device, consisting of both hydrophobic and hydrophilic materials, alternately deposited in a single, integrated build process, using a dual-material printer (Ultimaker 3). This created both hydrophilic and hydrophobic ducts in a single fabrication step, without the requirement for surface modification, or post-manufacture integration of different substrates. Using this approach, it was possible to produce water droplets in oil using
a hydrophobic PLA channel (Figure 1a), and oil droplets in water, within a more hydrophilic PVA channel (Figure 1b). In Figure 1a&b the aqueous wetting contact angle of PLA and PVA is depicted, together with the resultant droplet regimes. The increased hydrophilic properties of PVA enable inversion of the usual droplet formation regime to produce oil droplets in water. These material architectures were then combined in a single device, to sequentially produce water droplets in oil, followed by oil droplets in water, and finally, the ejection of water droplets from the device, to a collection bath (Figure 1c, Table S1, Video S1). This enabled the hierarchical assembly of complex, multiphase emulsions, using the monolithic microfluidic device, that was fabricated in a one-step process. Figure 1c-iii demonstrates the production of aqueous droplets (red) encapsulated within an oil droplet (blue), itself encapsulated within an alginate hydrogel shell (colourless) using this approach. These triple emulsions are shown to be sequentially deposited from the fluidic device, in effect, ‘printing’ individual complex emulsion droplets.

**Figure 1.** 3D-printing of microfluidic devices in hydrophobic and hydrophilic polymers enables the production of both water droplets in oil, and oil droplets in water. Dual-material printed microfluidic devices allow the sequential application of these droplet formation regimes, to produce triple emulsions of hierarchical water/oil/water droplet structures. a. 3D-printed polylactic acid (PLA) substrate displays hydrophobic properties demonstrated by water contact angle measurement. 3D-printed PLA T-junction generates water droplets in oil (schematic and experimental image). b. 3D-printed polyvinyl alcohol (PVA) substrate demonstrates more hydrophilic properties by water contact angle measurement. 3D-printed PVA T-junction generates oil droplets in water (schematic and experimental image of oil (blue) droplet ejection from PVA device into a water bath). c-i. Dual head extrusion printer enables fabrication of integrated PLA and PVA, dual material, microfluidic devices. c-ii. (Top) Schematic depiction of operational concept; sequential, droplet generating, flow-focusing junctions in PLA and PVA, enable the formation of double emulsions (water-in-oil-in-hydrogel (un-gelled) - w/o/w). Monolithic device realised in lower image, comprised of PLA (pink) and PVA (colourless) materials. c-iii. Triple emulsions form as hydrogel droplets are ejected from the microfluidic device. The outer, liquid, alginate phase is gelled upon dripping into a CaCl2 containing gelling bath, creating a triple water-oil-water emulsion with a gelled outermost shell. These complex emulsions (pink – aqueous internal phase, blue – oil mid-phase, colourless – outer alginate shell phase) are individually deposited from a dual material, microfluidic device. All scale bars: 1mm.
Characterisation of complex emulsion formation:
The formation of oil droplets in a continuous water phase, without surface modification, is usually challenging, owing to the native preferential hydrophobic wetting of many substrate materials, which favour the formation of water droplets in oil. Consequently, to date, only water droplet formation, in extrusion 3D-printed microfluidic devices, has been demonstrated [70]. We observe that in the formation of complex emulsions, the comparatively hydrophilic PVA junction is capable of operating in two distinct droplet generating regimes for the formation of oil droplets in water. These regimes are essentially, either dripping or jetting (Figure 2a), both being determined by the combination of input fluid flow rates, and the specific composition of the respective fluid phases (Figure 2b, Table S2). In the formation of triple emulsions, this results in the formation of either single, or multiple, encapsulated middle phase oil droplets (designated m), containing inner phase aqueous droplets (designated i) in the larger emulsion constructs, under jetting and dripping regimes, respectively. Figure 2b shows the manifestation of this with respect to the relative, and absolute, flow rates of the three input fluid phases, for both a fatty-acid rich sunflower oil, and a hexadecane/silicone oil mid-phase. The complex interplay of fluid flow properties, has been characterised in the determination of droplet formation, by dripping and jetting regimes and their transitions [71]. Fluid density, velocity, droplet and channel diameter, surface tension and viscosity, all play an important role, as drag and inertial forces compete with surface tension [72, 73]. It is unsurprising that this relationship becomes more complex in triple-phase systems, where flow rates govern both upstream droplet formation, and also influence linear flow velocity downstream at subsequent encapsulation. The fluid dynamics of droplet formation, are additionally perturbed by both internal, and external immiscible interfaces [74]. New dimensionless numbers have been proposed to describe triple emulsion droplet formation [75], albeit by a different microfluidic method with fluid phases unsuited to the creation of artificial cells. In this work, we take an empirical approach to control the number of inner (i) and middle droplets (m), that are encapsulated in the shell phase, by the tuning of fluid flow rates, to govern the droplet generation frequency, of each of the three phases. The output triple emulsion droplets have uniform morphology (Video S1), and relatively monodisperse size distributions (<4%) for each phase (Figure. S1). The size of the internal and mid-phase droplets is observed to change with the relative flow rates of the component fluid phases, corresponding with the internal and midphase droplet number. This is further detailed in supplementary figure S2.
Figure 2. Tuning fluid flows enables control of droplet morphology. 

**a.** The PVA junction responsible for oil in water droplet formation is capable of operating in two distinct droplet generating regimes in the formation of triple emulsions; either by dripping (1: left and top) or jetting (2: right and lower). These result in the formation of multiple (1: left and top), or single (2: right and lower), encapsulated middle phase oil droplets (blue), containing inner phase aqueous droplets (pink) within the larger emulsion construct (grey). Images (right) depict triple emulsion formation at the device exit, under dripping (top) and jetting (lower) regimes.

**b.** Ternary phase diagrams depicting flow-rate, and stacked column graphs detailing volumetric flow rates, of the three input phases and corresponding triple emulsion morphology. These detail the number of middle-phase droplets ($m$) and their number of constituent internal aqueous droplets ($i$), for operation with two different oil phases. On each plot, yellow illustrates two or more mid-phase oil droplets as a result of operation in a dripping regime, and green indicates a single mid-phase oil droplet from operation in a jetting regime. Index labels (a-j) enable cross-correlation between plots. Marker size on phase diagram denotes number of encapsulated inner aqueous droplets ($i$), with value quoted under stacked column graph (n=30 under each condition).

**3D microfluidic architectures enable formation of asymmetric and patterned complex emulsions, creating hierarchal polarised structures:**

The use of 3D printing enables the creation of fully 3D microfluidic architectures, unlike more traditional fabrication approaches, which are usually constrained to machining in a single plane. Using the extra-dimensionality afforded by 3D printing, allowed us to create devices which control the phase orientations and encapsulations within the complex emulsions system, thereby creating patterned and asymmetric droplet structures, around different geometric axes (Figure 3). The dominance of low Reynolds number flows, maintains the laminar streams of fluids, thereby minimising mixing. Axial symmetry at the droplet generating junctions was used to enable asymmetric fluid additions with respect to the major-channel axis. Whilst also creating a symmetric shear force on the disperse (droplet) phase. This maintained the patterning or organisation of emulsions, when the dispersed phase was pinched-off by the continuous (shell) phase. Complementary computational fluid dynamic modelling finds that the organisation within the resultant w/o/w emulsion flow, is stabilised in the extended fluidic duct in our experimental conditions (Table S3, Video S2). We found, that
whilst in flow, the inner aqueous droplets reached their equilibrium positions within the middle oil droplet, without ejection into the continuous hydrogel phase. This process is dominated by the viscous stress, competing with interfacial tension [76] (See supplementary discussion). Similarly, we find internal (i) droplets are constrained within lateral domains of the encapsulating oil droplet, as are differential additions of middle phase (m) oils, thus enabling the maintenance of internal hemispherical patterning and gradients (See Table S4 and supplementary discussion).

Using this experimental approach, we orientated sequential droplet generating geometries in 3D. This allowed us to spatially pattern the droplet structures with control over the designated patterned phase or phases, and the geometric orientation of patterning. Consequentially, this provided the ability to create both Janus shells, Janus and gradient mid-droplets (m), and achieve the controlled hemispherical placement of inner droplets (i). These may differ in size and contents, and be located in different regions within the encapsulating droplet (Figure 3). Figure 3a-i illustrates conceptually, how traditional 2.5D channel architectures can be used to create Janus shells with inner droplets as double emulsions. Enabled by 3D fabrication, it becomes possible to rotate the fluid inlets (by 90 degrees in this instance) providing the opportunity to create asymmetric co-flows, in different orientations with respect to the traditional single fabrication axis. In this way, it becomes possible to combine multiple co-flow orientations, creating both Janus shells, and Janus interiors, with independent and opposing orientations in both double (Figure 3a-ii), and triple (Figure 3a-iii) emulsions. In principle, this process can be extended to increasing hierarchies of droplet structures, in complex emulsions.

We also demonstrate that this approach can be extended to the production of differing inner droplets (i) in the triple emulsion, and dictate their location in the final droplet construct. This is achieved by joining two streams of water/oil emulsions, at a Y-shaped junction, to form a Janus flow, before its breakup into discrete encapsulated droplets (Table S1 row 3, Table S4). Figure 3a-iv illustrates the production of inner droplets (i) of two different sizes and contents (red ~430 μm and yellow ~125 μm) within a Janus encapsulating oil droplet (m), itself within a hydrogel shell. The smaller (yellow) inner droplets are retained to the right-hand perimeter of the larger red droplet population. With this approach to spatial patterning, enabled by 3D fabrication, in combination with the flow-rate controlling size and number of inner and middle-phase droplets (Figure 3b), we demonstrate that it is possible to produce a complex diversity of spatially compartmentalised and patterned, triple emulsion structures (Figure 3c) from these new 3D-printed, microfluidic devices. The outer shell phase is gelled on exit from the microfluidic device by the Ca\(^{2+}\) quickly diffusing and crosslinking the alginate shell in the gelling bath. As such, the final droplet structures are free-standing and able to withstand mechanical manipulation (Figure 3d). The alginate shell remains water permeable, thus enabling communicative access from the environment to the internal contents, where inner droplets can be segregated from the hydrogel shell by a bio-mimetic, lipid bilayer [59].
Figure 3. Three dimensional configurations of dual material fluidic channels, enable the spatial patterning of complex emulsions. a-i-iv. Four different fluidic channel geometries. The ability to manufacture channels and junctions in 3D-space (a ii-iv) provides new opportunities for the spatial patterning and organisation, of multiphase emulsions, compared to traditional planar (2.5D) channel arrangements (a-i). a-i. With fluidic channels constrained in a single plane, Janus-core and Janus-shell (bi-Janus) double emulsions, can be formed, with the hemispherical divide occupying the same orientation, in both instances. a-ii. Rotating the plane of one droplet generating flow focusing junction geometry by 90 degrees, results in the rotation of the plane of Janus droplet formation. Here, creating the depicted bi-Janus droplet hierarchies, with independent and opposing Janus orientation (90 degree rotation) in the core (mineral oil with and without oil blue N) and shell phases (alginate shell with and without silica particles or graphene oxide). a-iii. The addition of a fifth aqueous input (v) upstream of the consecutive hydrophobic and hydrophilic droplet generating geometries of a-ii, enables the addition of internal aqueous droplets (pink) as inner droplets in bi-Janus triple emulsions with perpendicular Janus patterning. a-iv. Extension of this principle to two independent upstream aqueous droplet generating geometries, combined to produce a parallel co-flow, enables the addition of two types (differing size and contents) of aqueous internal droplets within the resultant triple emulsion. The lateral offset delivery of these droplets into the common channel, defines the position of entry in the encapsulating droplet. Small aqueous droplets (yellow) are retained to the right-hand perimeter of the larger (pink) aqueous droplets within the Janus oil middle-phase of the triple emulsion. Complementary CFD modelling of the emulsion stabilisation within fluidic duct can be found in Table S4. b. Schematic illustration of parameter space, of droplet number and arrangement in triple emulsions (see also Figure 2). c. In combination, the spatial patterning enabled by 3D fluidic geometries demonstrated in (a) with the ability to control number of inner and middle phase droplets (b), a diverse range of complex and patterned triple emulsions can be made. (i and m subscript illustrates droplet numbers of construct). d. The encapsulating hydrogel shell, provides mechanical stability, thereby enabling physical manipulation of resultant emulsion droplets. Scale bars: 1 mm.

Functionalised encapsulated droplet interface bilayers (eDIBs).
In an immiscible, aqueous-oil system, phospholipids may serve as an effective amphiphilic surfactant, stabilising aqueous droplets in oil through the self-assembly of lipid monolayers at the water-oil interface. The contacting of two such interfaces leads to the spontaneous creation of a lipid bilayer, resembling the foundational structure of the cell membrane, and
membrane-bound organelles [50, 59]. In this way, membrane compartmentalised systems can be constructed within hierarchal droplet architectures, where functional membrane proteins can be reconstituted to enable both, the communication and transfer between different compartments, and, between compartments and the environment [59].

Expansion of the microfluidic patterning approach described above, was implemented for the creation of encapsulated, droplet interface bilayer systems with layered hydrogel shells. Within these constructs functional organic, and inorganic, materials were incorporated alongside lipid bilayers (Figure 4a, Video S3). In this way we produce encapsulated droplet interface bilayer systems (eDIBs, Figure 4b-i), with 25μm chemically exfoliated graphene oxide (GO) sheets incorporated into the outer shell at concentrations of up to 1.44% w/w (Figure 4b-ii). (GO characterisation Figure S2). GO is an atomically thin two-dimensional, functional nanomaterial that has attractive mechanical, electrical, chemical and photonic properties [77]. GO has been used to non-specifically bind proteins and peptides [78], thus it may be possible to serve as a protective shell layer to protect lipid bilayers from destabilising peptides and proteins, whilst retaining the capacity for water and small molecular diffusion, to the lipid bilayer. At higher concentrations, the presence of GO increased the viscosity of the hydrogel phase. Whilst this did not hamper the microfluidic formation of the hierarchical droplet assemblies, the slower surface-tension driven relaxation of the outer droplet, to a spherical shape during gelation, resulted in the gelation of tear-drop, or ovoid shaped, outer shells.

We also produced encapsulated droplet interface bilayer systems (eDIBs) with a functionally inert inner shell of osmotically matched buffer, followed by a second Janus outer-shell, containing embedded, 2μm porous magnetic silica microparticles. In this way a thin protective inner hydrogel region could serve as a spacer, in effect, isolating larger silica particles from direct contact with the lipid bilayers, but maintaining aqueous and diffusive continuity of the shell with the membrane. Silica particles have been reported to disrupt artificial bilayers [79] and we observed results consistent with this in the absence of the additional inner hydrogel layer. By the creation of two-layered shells, we could produce stable particle-laden encapsulated bilayer systems, with polarised properties, such as a Janus shell (Figure 4b-iii), or an asymmetric encapsulated droplet network (Figure S3). In these systems with inner (i) aqueous droplets, containing fluorophore sulforhodamine B, fluorescent imaging three days following manufacture, demonstrated that the lipid bilayer networks remained intact (Figure S4). The modulation of salt conditions of the alginate phase to avoid excessive osmotic stress on the assembled lipid bilayers, was also observed to slow the alginate gelation processes, giving rise to some shape fluctuation in the shell of the final constructs, as they gel on entry to the gelling bath, usually giving rise to slight elongation of the overall structure. It should be possible to expand our reported microfluidic methodology to employ in-channel gelation [59], to facilitate gelation whilst maintaining sphericity within the in-channel flow. The methodology reported here, provides a route for the fabrication of artificial cell chassis, containing both lipid membranes and functional nano- and micro-particulate materials, in spatially organised architectures. By using controlled spatial organisation, these materials may, therefore, harness the functionality of both the biological and non-biological components, whilst circumventing the physical incompatibilities, that would otherwise hamper realisation. In this way, designer hierarchal droplet structures, could be created to control bio-, chemical and physical properties.
**Figure 4.** In the presence of phospholipids, droplet networks segregated by lipid bilayers may be formed within a triple emulsion. This creates encapsulated droplet interface bilayer networks (eDIBs), that can be patterned with functional materials using the fluidic patterning approach illustrated in Figure 3. **a.** The presence of amphiphilic phospholipids in the oil mid-phase, serves as a surfactant, forming self-assembled lipid monolayers, at each oil-water interface. The contacting of two such aqueous interfaces, results in the formation of a lipid bilayer between the aqueous volumes, creating lipid membranes between internal aqueous droplets, and the internal droplets and the hydrogel shell, where they make contact (b-i). Incorporation of two successive outer alginate co-flows, in emulsion formation, can be used to form double-layered hydrogel shells. These can be use to encapsulate droplet networks with Janus-, or whole-shell-, patterning that incorporates functional materials, such as graphene oxide or silica magnetic particles. **b-ii.** Brightfield and fluorescent images of encapsulated droplet interface bilayers (eDIBs), with an outer hydrogel shell loaded with atomically thin graphene oxide (GO) sheets. **b-iii.** Encapsulated droplet interface bilayers (eDIBs) with a double hydrogel shell, comprising an inert spacer shell, and an asymmetric Janus-shell. The Janus shell incorporates silica magnetic microparticles to impart polarised magnetic properties on the membrane-based artificial cell construct.

**Polarisation induced properties of artificial cell chassis:**
The particle-containing encapsulated bilayer systems, can gain specific functionality from patterned and encapsulated components. Here we demonstrate the polarisation induced functional properties, of the encapsulated droplet interface bilayers (eDIBs) complete with an outer Janus shell of embedded, paramagnetic, silica microparticles. The incorporation of paramagnetic particles, enables the manipulation of the eDIBs, by an external magnetic field, enabling mobility and orientational control, in aqueous environments (Video S4). These artificial cell chassis can be moved individually in an aqueous environment (Figure 5a). In a rotating magnetic field, individual constructs orientate with respect to the magnetic field and to the polarity of their Janus shell, thereby providing a directionality to the droplet structure. These behaviours could be combined in an aqueous pool, where Janus shell eDIBs could; seek, orientate, move, and submerge to interface with a static magnetic target placed beneath the Petri-dish. The preferred orientation of the construct is governed by the polarised patterning of the eDIB structure (Figure 5c). Such polarisation induced properties, created by the ability to spatially organise and pattern eDIBs, provides the opportunity to recapitulate biological and biological-like behaviours. These may include the harnessing of polarisation in directional control, polarised chemical organisation, or the exploitation of anisotropy, for example in the self-organisation of higher-order assemblies or artificial tissues. Figure 5d shows a population...
of core-shell Janus magnetic architectures, in an aqueous environment under a rotating magnetic field. All constructs are observed to spontaneously orientate such that their hemispherical division is in the vertical plane. Individual constructs are observed to migrate clockwise, whilst simultaneously rotating about their own central axis. This is a consequence of both the overall, and locally polarised, magnetic properties of each construct. Colliding constructs are observed to temporarily co-migrate with inhibited rotational velocity, before separation and recovery of rotational motion (Figure 5d-ii, Table S4 correlation test). The orientation of individual constructs may all be unified with this type of patterning, and their motion within a larger population may influence others within the group [25]. Such mobility synchronisation results in the emergence of simple, group level, behaviours. Thus, these principles may serve to pave the way for the design and programming of more sophisticated, population level behaviours, governed by polarised or directional functionality, in membrane-based artificial cell communities.

Figure 5. Polarised encapsulated droplet interface bilayers (eDIBs), with an asymmetric Janus-shell of silica magnetic microparticles, exhibit directional locomotion in an aqueous environment. a. A single magnetically polarised eDIB is corralled from a population with a non-contacting magnetic wand. b. Time-sequence of images, of a magnetically polarised eDIB, orientating with respect to a rotating magnetic field. The construct rotates clockwise about its central axis. The dotted line indicates the radial boundary of the magnetic and non-magnetic hemispheres. c. Time-sequence images of magnetically polarised eDIB orientating and migrating in three dimensions to a magnetic target beneath the Petri dish. c-i. Orientation in the plane to face the magnet. c-ii. Migration on the surface plane. c-iii. Re-orientation of the magnetic hemispherical-face towards the magnet and submersion of the construct. c-iv. Localisation at lower surface of the Petri dish at magnetic target location. d. A population of core-shell, Janus constructs, containing magnetic microparticles in one hydrogel hemisphere, collectively migrate and spin in a rotating magnetic field. d-i. Tracked trajectories (subset shown for clarity) highlight clockwise migration about a central point. All constructs adopt a common axial orientation, and also spin clockwise about this axis whilst migrating. d-ii. The directionality of each construct is observed by the polarised hemispherical face. Colliding constructs experience a shared reduction in angular rotational velocity, temporarily before moving apart, and restoring velocity (See also Video S4). Scale bars: 1 mm.
Discussion:
In summary, we have demonstrated the feasibility of a new, simple, one-step process, that uses 3D printing to fabricate monolithic, dual-material, microfluidic devices, which are able to produce both water-in-oil, and oil-in-water, droplet emulsions, without the need of surface modification. With this approach to device fabrication, we demonstrate the sequential operation of hydrophobic and hydrophilic droplet generating geometries, to create hierarchal droplet structures, where flow rates determine the mode of operation, and, govern the number of encapsulated inner and mid-phase droplets. By exploiting the ability to completely fabricate manifolds in three dimensions, we are able to generate triple emulsions with 3D patterned morphologies. This provides the capability to produce, not only Janus shell materials, but hierarchical double-Janus (shell and mid-phase) droplets, with independent control of the hemispherical orientations. These features can be combined with inner droplet populations of differing size, number and hemispherical location, alongside the ability to create layered and patterned shells. These droplet templates are able to incorporate lipid bilayers, providing membranes between neighbouring aqueous droplets, and between inner droplets and their contact of the mid-phase-and-hydrogel interface. This provides the opportunity to harness lipid membrane, and membrane protein biochemistry, together with biochemical compartmentalisation and environmental communication. We are able to combine the presence of lipid membranes with the integration of functional organic and inorganic materials, patterned within the same hydrogel matrix shell. The hydrogel encapsulation provides mechanical rigidity and aqueous compatibility, whilst microfluidic manufacture provides a potential means to scale manufacture, through parallelisation of capillary structures [57,58]. 3D microfluidic architectures constructed from dual hydrophilic and hydrophobic materials, enables the control of the landscape of hierarchal droplet construction, and provides the ability to pattern the morphologies of the droplet hierarchies, and break axial symmetry. This enables the creation of polarised droplet structures, which can be further used to impart additional functionality. This is demonstrated here for the first time, via the construction of hemispherically-magnetic, encapsulated droplet interface bilayers, enabling controlled orientation, locomotion and rotation.

The rapid, dual-material, fabrication process, means that device designs can be rapidly iterated and customised for different desired droplet arrangements. Something not previously possible. This in effect, creates the possibility of disposable devices for complex emulsion production, or even use as cartridges for droplet printing. Using 3D channel fabrication techniques, it is conceivable that non-homogeneous, but spherically centrosymmetric, droplet hierarchies may be produced via a radial array of droplet inlet channels into the pre-encapsulating flow. Current work is directed towards improved understanding of precise inner-droplet arrangements to further increase design control. In the reported work, the new use hydrophilic PVA serves to demonstrate feasibility of the dual material approach, reporting the creation of complex emulsions using a fused-filament 3D printed device, for the first time. However, ultimately, the hydrophilic PVA material is non-optimum, as it is partially soluble in aqueous media, in this case the hydrogel shell phase, over prolonged periods of use. In the immediate-term, this time-limited use may be offset by the rapidity of device manufacture. Whilst the current commercial palette of printable polymeric materials is limited, it is rapidly growing [80]. Therefore, the prospect of further hydrophilic polymer substrate materials is promising, as 3D printing enjoys continued growth across ever widening application areas. Similarly, advances in printer resolution can be expected to facilitate the production of narrower channel architectures, thereby enabling the miniaturisation of patterned emulsions. With the growing interest in 3D printing of soft-materials [81], the ability to deposit stable patterned, complex emulsions from a 3D printed device, as demonstrated here, may serve as
the basis for the 3D printing of large-scale materials, with complex emulsions or artificial cells as the constituent building blocks. This may find use in bioprinting applications, or in the construction of increasingly complex artificial tissues made from populations of synthetic cells, affording increased material complexity, in comparison to single phase droplet printing [20]. The reported spatial patterning of complex emulsions could be applied with incorporated living cells, enabling the concept of bio-hybrid materials, which could combine membrane bound droplets and cells [82], or the integration of cells into hydrogels [70, 83]. The ability to impart polarisation or directional preferences, in cell-laden constructs, may find use in the self-organisation of 3D tissue engineering and repair systems. The engineering of orientation-dictated, higher-order assembly of artificial tissues from component cells, holds significant promise. Likewise, polarisable artificial cells, may take advantage of directional-specific behaviours, analogous to phototropic or gravitropic behaviour in plants, albeit by alternative mechanisms. The ability to create soft-matter Janus materials with integrated biological components also paves the way for applied uses similar to their solid-state counterparts, such as in sensing or displays, but with integrated bio-chemical capabilities.

Droplets, lipid bilayers, soft-matter compartmentalised structures, and functional nano- and micro-materials, are all the subject of great interest as building blocks of bio-inspired engineered materials. The ability to create spatially organised and patterned droplet structures, comprising all these elements within the same entity, will likely serve to increase the sophistication of artificial cell models, as functional materials combining biological, organic and inorganic material properties. The ability to impart asymmetric, polarised or gradient patterning on such hierarchical droplets in any desired orientation, at their formation, through the use of 3D printed microfluidics, affords a new level of control in the design and functional utility of these materials. Biology routinely exploits the emergent functionality of the barrier properties of membranes, in combination with chemical gradients and polarisation, to impart directional or symmetry-breaking properties in cells, and enable subsequent individual and collective functionality. This serves as inspiration for the next generation of dynamic, functional, synthetic materials, built using these approaches. The data presented here, demonstrates the capability to engineer chemical patterning and impart such properties in aqueous compatible, hydrogel-encapsulated, membrane-bound droplet networks. This holds significant promise for the advancement of behaviours and functionalities that more closely mimic the sophisticated functionality of biological cells. These patterned, hierarchical droplet materials, are operational in aqueous environments, in which they remain free-standing, and retain diffusive communication between the environment and internal membrane-bound architectures. This renders them promising candidates for applications across the life-sciences, both within and outside the laboratory, such as for responsive drug delivery, self-repairing and re-configurable materials, and biochemical computation.

**Experimental Section:**

**Chemicals and components**

Alginic acid sodium salt, calcium chloride, sodium chloride, silicone oil AR20, hexadecane, chloroform, Oil Blue N, PVA powder, tween 20, and span 80 were purchased from Sigma-Aldrich. Sunflower oil (Pure) was purchased from Sainsbury’s. Hydrophilic, magnetic silica microparticle (SiMAG/MP-DNA 2.0um) was purchased from Chemicell. 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) lipid was purchased from Avanti Polar Lipids. Sulphorhodamine B was purchased from Sigma Aldrich. Neodymium magnets were purchased from RS Components. Large flake size of graphene oxide suspension was synthesized using a method previously reported by Rocha et al. elsewhere [84]. The concentration of GO in the water (1.44wt%) was calculated by weight difference after freezing and freeze drying (48h) 4-6 grams of suspension. Triple emulsion templates are
shown in Table S1. The preparations of the precursor for the eDIBs formation were as in our previous work [59]. Calcium chloride solution (0.5M) was prepared for the outer alginate matrix gelation. To prepare the microparticle containing alginate solutions, the original particle-containing solution was diluted with deionized water (1% v/v for SiMAG/MP-DNA, and up to 1.44wt% for GO slurry), and the alginate powder was added to prepare a 3wt% alginate solution using a magnetic stirrer (IKA RCT basic safety control) agitated at 800rpm and 50 degrees Celsius for 4 hours. The solution was stored at 4 degrees Celsius.

**Triple Emulsion Construct Materials**

Internal aqueous phase contained 0.5M NaCl with 100mM sulphorhodamine B (pink) for observation. The oil mid phase for eDIB formation is formulated as hexadecane and silicone oil AR20 (1:2 v/v) with DPhPC 8.33 mg/ml for droplet network production. The oil phase for non-bilayer constructs were formulated with either sunflower oil, or mineral oil, with surfactant (see Table S2). Oil Blue N was added at 0.05wt% for visualisation, to demonstrate liquid Janus cores, visualise internal concentration gradients, and their persistence throughout and beyond the microfluidic production process. Shell phases were formulated with 3% alginate solution, containing additional 0.5M NaCl (for the eDIB inner alginate shell to osmotically match the internal aqueous phase droplet), or GO or silica nanoparticles (for the eDIB outer alginate shell patterning) as described in the text. The gelling bath contained 0.5M CaCl₂ solution.

**Microfluidics**

Microfluidics devices were designed and modelled with COMSOL Multiphysics (the dimensions of tubular channels are in the range of 240μm to 1200μm diameter), and were printed using fused filament fabrication printers (Ultimaker 3) with PLA filaments and PVA filaments (Ultimaker), using 0.4mm AA and 0.4mm BB print-cores. The print g-codes were programmed using Cura (version 3.3.1) software with customised settings (Figure. S1). The layer height was controlled at 0.06mm, and the printing speed was tuned at 100mm/s with default printing temperatures (200 degrees Celsius for PLA filaments and 215 degrees Celsius for PVA filaments). The printed parts were treated with chloroform vapour within an enclosed metal box for 8 minutes, and the treated pieces were left in a fume cupboard for two hours before use [70]. Precursors were loaded in syringes (5mL, gas-tight, SGE Analytical Science), and were delivered to the microfluidic devices at a constant flow-rate, through PEEK and PFA interconnects and FEP tubing, using syringe displacement pumps (KD Scientific, model 789200L). Details of the controlled sequential emulsification using multiphase microfluidics, is given in our previous work [22].

**Experiments and measurements**

General images and videos were taken using a 12MP digital camera with mounted zooming lenses, on 3D printed stands. Epifluorescence and light micrographs were captured using a modified Nikon Eclipse Ti-U inverted microscope and Andor iXon ultra 897 EMCCD camera. For white light images, illumination was provided by the microscopes integrated 100W halogen lamp, while a Shanghai Dream Lasers 532nm DPSS laser with a power output of 100mW, was utilised for epifluorescence illumination. Laser coupling into the microscope was achieved via a custom-built optical circuit utilising components sourced from Thorlabs Chroma and Semrock followed by a single mode fibre-optic launch. A low magnification 1x (Nikon Plan UW) objective was used in all acquisitions. Excitation and emission fluorescence wavelengths were separated using a 532nm edge dichroic mirror combined with a 542nm edge long-pass filter and a 565-615nm bandpass filter. Two colour overlays were generated using the FIJI distribution of ImageJ. Particle coordinates data were collected and analysed Vernier Video Physics, (version 3.0.5). Image processing was performed using ImageJ. Computational fluid dynamic modelling was done in COMSOL Multiphysics (version 5.4), using moving mesh method.

**Data analysis**
See SI for GO characterisation data and methods.

Droplet Rotation Analysis: The raw data of the moving droplets was evaluated and processed using IBM SPSS software. The linear relationships of the droplets’ angular displacement profiles were analysed using a Pearson product-moment correlation test.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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J.L., D.A.B. and O.K.C. conceived the research. J.L. designed and performed all the experiment. D.K.B. contributed to the eDIB formation experiments. W.D. J. conducted the fluorescence imaging. V.G.R contributed to the graphene oxide synthesis, modification and characterisation. W.X contributed to the SPSS data analysis. J.L., D.A.B. and O.K.C. wrote the manuscript. All authors discussed the results and commented on the manuscript. The authors declare that they have no competing interests. This work was primarily supported by an MRC Confidence in Concept award to O.K.C. who led the research. O.K.C. also acknowledges funding from Wellcome Trust ISSF in support of D.K.B. and W.D.J.. V.G.R acknowledge the EPSRC Grant Graphene 3D Networks (EP/K01658X/1) and would like to thank the European Commission (FP7—Marie Curie Intra-European Fellowship GRAPES).

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