A floating mould technique for the programmed assembly of protocells into protocellular materials capable of non-equilibrium biochemical sensing

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ABSTRACT: Despite important breakthroughs in bottom-up synthetic biology have recently been achieved, a major challenge still remains the construction of free-standing, macroscopic and robust materials from protocell building blocks that are stable in water and capable of emergent behaviours. Herein we report a new floating mould technique for the fabrication of millimetre- to centimetre-sized protocellular materials (PCMs) of any shape that overcomes most of the current challenges in prototissue engineering. Significantly, this technique also allowed us to generate 2D periodic arrays of PCMs that displayed an emergent non-equilibrium spatiotemporal sensing behaviour. These arrays were capable of collectively translating the information provided by the external environment and encoded in the form of propagating reaction-diffusion fronts into a readable dynamic signal output. Overall, our methodology opens up a route to the fabrication of macroscopic and robust tissue-like materials with emergent behaviours, providing a new paradigm of bottom-up synthetic biology and biomimetic materials science.

Introduction

Living tissues comprise complex three-dimensional (3D) architectures of interconnected cell consortia that communicate and display emergent behaviours. Mimicking the structure of living tissues and understanding the physical-chemical basis of their emergent properties are two of the major goals of bottom-up synthetic biology. Their achievement will lead to important technological advancements in tissue engineering, pharmacokinetics, personalised therapy, micro-bioreactor technologies, and soft robotics.[1-6]

In recent years, while working towards these goals, researchers in the field of bottom-up synthetic biology started to develop methodologies to assemble different models of *synthetic protocells*[7-13] into interconnected 3D networks, termed *prototissues*, that communicate and display rudimental emergent behaviours.[6] For example, H. Bayley and co-workers developed a 3D printing technique to pattern water-in-oil micro-droplets connected by interface bilayers (DIBs) into synthetic tissues. They then demonstrated that DIBs are capable of membrane protein-mediated electrical communication, macroscopic deformation, and light-induced gene expression.[14-16] Q. Li *et al.* used magnetic fields to manipulate diamagnetic giant unilamellar lipid vesicles (GUVs) into various spatially coded configurations of a few hundred micrometres in size.[17] X. Wang *et al.* showed that micro-arrays of hemi-fused GUVs could be patterned using acoustic standing waves, thus making progress towards the fabrication of prototissues with controlled geometries and lattice dimensions.[18] Instead of patterning using 3D printing or by applying magnetic fields or acoustic standing waves, our group has recently developed a synthetic approach to the programmed assembly of prototissue spheroids based on the interfacial bio-orthogonal adhesion of two populations of reactive protein–polymer protocells, termed *proteinosomes*.[19] Proteinosomes are a well-established protocell model and are generated using the Pickering emulsion technique. They comprise a semi-permeable and elastic membrane which consists of a closely packed monolayer of conjugated bovine serum albumin/poly(N-isopropylacrylamide) (BSA/PNIPAM) amphiphilic nanoparticles, and because of this they can be classified as organic colloidosomes. The BSA/PNIPAM membrane is then chemically crosslinked with PEG-bis(*N*-succinimidyl succinate) (PEG-diNHS) and the proteinosomes can be transferred into water media. Most importantly, proteinosomes can be engineered to display protocellular properties such as guest molecule encapsulation, selective permeability, gene-directed protein synthesis, membrane-gated internalized enzyme catalysis, predatory behaviours, and reversible contractility.[20-25] To assemble proteinosomes into prototissue spheroids, we first synthesized a new BSA/poly(N-isopropylacrylamide)-co-methacrylic acid (BSA/PNIPAM-co-MAA) nanoconjugate and functionalised it with either pendent azide or bicyclononyne (BCN) moieties. The amphiphilic bio-orthogonally reactive protein-polymer nanoconjugates were then used to prepare two separate populations of azide- or BCN-functionalised proteinosomes as water-in-oil (w/o) droplets using the Pickering emulsion technique. The proteinosome structures were stabilised *via* chemical crosslinking with PEG-di NHS, which was pre-dissolved in the water phase. Binary populations of the azide- and BCN-functionalised proteinosomes were then spatially confined using a water-in-oil-in-water (w/o/w) Pickering emulsion procedure and structurally interlinked *in situ via* an interfacial strain-promoted alkyne-azide cycloaddition (I-SPAAC) reaction to afford prototissue spheroids 75–200 μm in diameter upon removal of the inner oil phase.[19]

While all these different approaches provided important breakthroughs in prototissue design and synthetic construction, they are not without their drawbacks. The w/o/w Pickering emulsion method does not provide spatial control over the protocell organisation and is currently limited to the generation of prototissue spheroids with micrometre-scale dimensions;[19] acoustic patterning requires the standing waves to be constantly applied to avoid a rapid re-dispersal of the GUVs into the bulk

solution;[18] the diamagnetic GUVs require an aqueous media containing high levels of $MnCl_2$ and a constant magnetic field to maintain the patterns;[17] and the 3D-printing of DIBs requires the presence of an external bulk oil phase and the resulting prototissues present a very short shelf life.[26] As a consequence, the possibility of using protocells as building blocks to assemble macroscopic materials that are robust, free-standing, characterised by complex internal 3D architectures, capable of communicating both internally and with the external environment, and displaying emergent behaviours that generate from the synergistic interaction of their constituent parts still remains a considerable challenge. The development of such *protocellular materials* (*PCMs*) would open up new avenues in bottom-up synthetic biology and bioinspired engineering and facilitate the transition of protocell research from fundamental to applied science.

As a step towards this ambitious goal, in this article we describe the first bottom-up methodology for the fabrication of PCMs that overcomes most of the current challenges in prototissue engineering. This methodology is based on a floating polytetrafluoroethylene (PTFE) mould, which can be used for the programmed assembly of millions of bio-orthogonally reactive synthetic protocells into centimetre-sized free-standing tissue-like materials of any size and shape. These PCMs are stable in water media and are capable of communicating both internally and with the external environment. Significantly, this novel floating mould technique could also be used to generate for the first time 2D periodic arrays of PCMs, which were capable of an emergent non-equilibrium spatiotemporal sensing behaviour. These arrays were capable of dynamically translating the information provided by the external environment and encoded in the form of propagating reaction-diffusion gradients into a readable signal output. In general, our work moves beyond the engineering of a strategy to generate protocell-protocell adhesions. It aims to spearhead the programmed assembly and spatial integration of different protocell phenotypes into centimetre-sized free-standing PCMs with precise architectures and geometries. Thanks to these unique characteristics, the PCMs can then combine the specialisation of individual protocell types with the emergent spatiotemporal biochemical response of the ensemble, thus providing a new paradigm of bottom-up synthetic biology and biomimetic materials science.

Results and Discussion

Programmed assembly of protocellular materials (PCMs). Protein-polymer PCMs were generated from a binary population of bio-orthogonally reactive proteinosomes in oil. First, RITC-labelled azide-functionalised BSA/PNIPAM-co-MAA nanoconjugates (red fluorescence) and FITC-labelled BCN-functionalised BSA/PNIPAM-co-MAA nanoconjugates (green fluorescence) were synthesised using our previously established procedure.[19] Subsequently, samples of RITC-labelled azide- and FITC-labelled BCN-functionalised proteinosomes in oil (mean diameter *ca.* 25 μm; mean volume *ca.* 8 pL) were prepared using the Pickering emulsion technique and internally crosslinked with PEG-diNHS (Scheme 1a, Supplementary Figure S1, Supplementary Section 1.2).[19-20] Subsequently, the two populations of crosslinked RITC-labelled azide- and FITC-labelled BCN-functionalised proteinosomes in oil were mixed in a 1:1 ratio and dropcasted inside a circular PTFE mould 2 mm in diameter floating on an aqueous solution of polysorbate 80 (5 wt%) to obtain a final emulsion volume of 0.64 μL mm^{-2}. The binary emulsion was then allowed to transfer to the water media for *ca.* 2 hrs with an associated progressive colour change from white to transparent (Scheme 1b, Supplementary Section 1.3). Time-dependent fluorescence microscopy imaging showed that PCMs formed *via* a progressive oil removal process with concomitant bio-orthogonal ligation of the binary proteinosome population (Supplementary Video 1, Scheme 1c). This resulted in membrane-bounded PCMs with a spatially integrated tissue-like structure

that remained attached to the PTFE mould, as shown by fluorescence microscopy imaging (Figure 1a). Confocal fluorescence microscopy showed that the PCMs had a homogeneous thickness of *ca.* 180 µm (Figure 1b,c). Significantly, the connection between the PCM and the PTFE mould was strong enough to allow for the mould to be lifted from the aqueous solution and for the sample to be handled in air (Figure 1d). The PCM could also be easily detached from the mould, resulting in free-standing PCMs in water media that could be manipulated with tweezers. This allowed for the preparation of samples for SEM imaging, which showed tightly interconnected protein-polymer cell-like structures resembling plant tissue or squamous epithelium tissue, highlighting the free-standing nature of the material (Figure 1e, Supplementary Figure S2).

Due to colour change from white to transparent, the oil removal and PCM programmed assembly process could be monitored using a digital camera to obtain PCM transfer curves as a function of the emulsion volume used to assemble the PCMs, ranging between 0.16 and 0.64 µL mm^{-2} (Supplementary Section 1.4, Supplementary Figure S3, Supplementary Video 2). The PCM transfer curves displayed a sigmoidal shape, and the onset time of the curve was found to increase linearly with the emulsion volume used (Figure 1f, blue plot). In contrast, the final PCM transfer time, defined as the intersection of the slope of the sigmoidal curve and the plateau region, displayed a logarithmic growth (Figure 1f, orange plot). These observations seem to indicate that the onset of the oil removal process depends linearly on the volume of 2-ethyl-1-hexanol present in the sample, but the rate of diffusion of the oil into the bulk solution tends to reach a threshold value at high emulsion densities. Moreover, the importance of polysorbate 80 in the oil removal and PCM programmed assembly process was also highlighted by control experiments carried out in the absence of the surfactant. Under this condition a strong osmotic pressure across the PCM caused the growth of large water bubbles on top of the PCM with concomitant PCM deformation and rupture when the bubbles reached a critical size (Supplementary Video 3). This still allowed for a slow transfer of the prototissue into water (*ca.* 8 hrs), but the resultant PCM proved more fragile and inhomogeneous.

Significantly, control experiments carried out using non-bio-orthogonally reactive proteinosomes highlighted the critical role of bio-orthogonal chemistry in the PCM generation. Supplementary Video 4 compares the oil removal and PCM programmed assembly process of two experiments performed in parallel using normal (non-bio-orthogonally reactive) proteinosomes (left) and bio-orthogonally reactive proteinosome (right). In the absence of bio-orthogonal ligation the Marangoni flow pushed proteinosomes to the edge of the mould and dragged them into the bulk solution (Scheme 1), resulting at best in the formation of a thin and defective PCM (Supplementary Video 4, left). In contrast, in the presence of bio-orthogonal ligation, as soon as the oil was removed and azide- and BCN-functionalised proteinosome entered in contact, they promptly reacted *via* the I-SPAAC reaction and formed a spatially integrated tissue-like structure (Supplementary Video 4, right).

Taken together, these observations seem to indicate that the PCM assembly process involves a synergistic effect of surfactant-mediated oil removal, Marangoni flow, and bio-orthogonal ligation. Most importantly, our new floating mould technique allows us to assemble proteinosome building blocks together into macroscopic and free-standing PCMs that are mechanically robust and stable in water media for months.

Generation of PCMs with complex 3D architectures. Having established that the floating mould method can be successfully used to generate macroscopic and free-standing tissue-like materials from a binary

community of bio-orthogonally reactive proteinosomes, we next explored its versatility for the generation of PCMs with complex 3D architectures.

First, we explored the possibility of generating PCMs of different shapes and sizes. As a step towards this goal, we built a PTFE mould in the shape of an equilateral triangle with 1.0 cm sides and a PTFE mould in the shape of a square with 0.5 cm sides and used them to generate PCMs at a 0.64 µL mm^{-2} emulsion volume. Epifluorescence microscopy images showed the successful programmed assembly of defect-free PCMs in the shape of a triangle (Figure 2a) and of a square (Figure 2b) of the desired dimensions. Neither of the PCMs presented cracks or defects, both fully transferred to the water phase and remained attached to the PTFE mould. Despite the large area (43.3 and 25.0 mm^2, respectively), both PCMs could be easily lifted from the surfactant solution using the mould and transferred to a different solution without breaking. Significantly, since the PCM shape is defined by the PTFE mould and this can be laser-cut using a high-precision CNC machine, there is virtually no limitation to the complexity of PCM shapes that can be fabricated with this method. To showcase this, we constructed different PTFE moulds with our research group's logo (size: 6.5 x 2.2 cm) using 3 different text fonts and used them to assemble PCMs with complex shapes. Epifluorescence microscopy imaging showed the successful programmed assembly of millions of RITC- and FITC-labelled bio-orthogonally reactive proteinosomes into the Gobbo Group's logo (Figure 2c, Supplementary Figures S4-6).

Next, we explored the possibility of generating patterns of different proteinosome populations within the same PCM. To achieve this, we synthesised 4 different populations of BCN- and azide-functionalised proteinosomes in oil: 1) non-labelled azide-functionalised proteinosomes, 2) Dylight405-labelled BCN-functionalised proteinosomes, 3) FITC-labelled BCN-functionalised proteinosomes, and 4) RITC-labelled BCN-functionalised proteinosomes. These 4 populations were then used to generate 3 binary populations of proteinosomes by mixing the non-labelled BCN-functionalised proteinosomes in a 1:1 ratio with the Dylight405-, FITC-, or RITC-labelled azide-functionalised proteinosomes. Patterned PCMs with circular and concentric green and red fluorescent proteinosome populations on a background of blue fluorescent proteinosomes were then generated by manual patterning of the 3 differently labelled binary populations of proteinosomes in oil inside a 0.5 cm wide square PTFE mould using a mechanical pipette. The patterned emulsions were then allowed to transfer to the aqueous phase and assemble into the patterned PCM (Supplementary Information Section 1.5). Epifluorescence microscopy imaging showed successful formation of the desired PCM with circular concentric patterns of proteinosome consortia (Figure 2d). Most importantly, no noticeable differences were observed in the pattern when the PCM was flipped upside-down and imaged (Supplementary Figure S7). This indicated that the technique produced patterns that were homogenous through the PCM thickness, that is, the pattern remained in the xy plane and no stacking of the proteinosome populations was observed.

Stratified PCMs could then be generated using a layer-by-layer technique. First, we cast a binary population of Dylight405-labelled bio-orthogonally reactive proteinosomes in oil and allowed them to transfer into water and assemble into a first PCM layer *ca.* 90 µm thick. Other 2 proteinosome layers could then be cast on top this first layer simply by repeating the same protocol using different fluorescently labelled binary populations of bio-orthogonally reactive proteinosomes in oil. Upon transfer, each proteinosome layer adhered to the layer underneath *via* an inter-layer I-SPAAC reaction, resulting, overall, in a stratified prototissue *ca.* 270 µm thick. Confocal fluorescence microscopy imaging showed that all layers were homogenous in thickness and no layer delamination was observed (Figure 2e, Supplementary Figure S8).

Overall, these results demonstrate the high versatility of our floating mould technique. Our novel approach is extremely promising and pioneers a route to the design and synthetic construction of PCMs of large size and of any shape that are stable in water media and comprise patterns and layers of different protocell consortia.

Non-equilibrium biochemical sensing in PCMs. Inspired by the above observations, we extended our methodology to construct the first PCMs capable of supporting a collective and coordinated spatiotemporal biochemical response *via* internally derived molecule-based signalling. As a first step towards this goal, we investigated whether the PCMs were capable of sensing the external environment and triggering a coordinated internalised cascade of chemical signals *via* enzyme catalysis. To achieve this, we prepared a circular PCM 2 mm in diameter from a binary population of FITC-labelled BCN-functionalised and non-labelled azide-functionalised proteinosomes that were preloaded with glucose oxidase (GOx) and horseradish peroxidase (HRP), respectively (Supplementary Section 1.7, 1.8). Once transferred to the water media, the PCM was moved to a Petri dish containing an aqueous solution of glucose (Glc, 20 mM) and Amplex Red (0.5 mM). This initiated a spatially coupled GOx/HRP enzyme cascade reaction under diffusional equilibrium conditions between the 2 bio-orthogonally interlinked protocell populations. The GOx containing protocells converted Glc to D-glucono-1,5-lactone (GDL) and internally produced the signalling molecule H_2O_2, which was used to communicate to the HRP-containing protocells to oxidise Amplex Red to resorufin and produce a fluorescent signal (Figure 3a). Epifluorescence microscopy was used to determine the location of the GOx-containing FITC-labelled BCN-functionalised proteinosomes within the PCM and monitor the onset and *in-situ* development of red fluorescence due to the endogenous production of resorufin (Figure 3b, c, Supplementary Video 5). Typically, the onset of red fluorescence in the HRP-containing protocells occurred within the first minute, followed by diffusion into the neighbouring GOx-containing protocells and external environment. By contrast, control experiments involving PCMs lacking either GOx or HRP showed no fluorescence increase due to the inability of these PCMs to sense glucose in the surrounding environment or synthesize resorufin, respectively (Figure 3c).

Given the PCM's ability to sense and respond to chemical changes in the environment, we next showcased the potential of our floating mould technique by assembling, for the first time, 2D arrays of spatially encoded PCMs that could detect and visualise advancing concentration fronts of chemical gradients under non-equilibrium conditions. To achieve this, we first constructed a new PTFE mould featuring a 4x4 array of circular wells for PCM production and an injection point placed on the left-hand side of the array (Figure 4a). This PTFE mould was utilised to assemble a 4x4 array of enzymatically active circular PCMs 2 mm in diameter (Supplementary Section 1.9). Each PCM comprised an interlinked 1:1 binary population of non-labelled GOx-containing BCN-functionalised proteinosomes and HRP-containing azide-functionalised proteinosomes. Subsequently, the PTFE mould loaded with the array of PCMs was let to float on 0.980 mL of a phosphate buffer solution (PBS, 10 mM, pH 6.8). The PCM array was then exposed to a left-to-right unidirectional reaction-diffusion gradient by injecting 20 µL of an aqueous solution of glucose (Glc, 100 mM) and *o*-phenylenediamine (*o*-PD, 50 mM) through the injection point on the left-hand side of the PCM array. This induced spatiotemporal oxidase/peroxidase responses to the co-diffusion of Glu and *o*-PD substrates across the periodically ordered enzymatically active PCM array. The array's spatiotemporal responses could be followed using time-dependent fluorescence microscopy by monitoring the development of green fluorescence associated with the HRP-mediated oxidation of *o*-PD to 2,3-diaminophenazine (2,3-DAP) in the azide-functionalised proteinosomes. In general, a wave of 2,3-DAP production unidirectionally moved across the PCM array from left to right. We associated this with the progressive co-diffusion of Glc and *o*-PD substrates (Figure

4b, Supplementary Video 6). Time-dependent mean fluorescence intensity analysis showed that the onset time of fluorescence (OT) increased quadratically across the rows of the array oriented parallel to the substrate diffusion front (Figure 4d), whereas it showed minimal difference across the columns oriented perpendicularly to the substrate diffusion front (Supplementary Table S2, Supplementary Figure S9). A similar trend was found for the initial rates of 2,3-DAP production, which gradually diminished across the rows placed parallel to the direction of the substrate diffusion front and showed comparable rates across the columns placed perpendicular to the substrate diffusion front. The fluorescence associated with each individual PCM was found to increase to a steady state and then slowly decrease in intensity. We attributed this to the consumption of the substrates and to the diffusion of the 2,3-DAP into the bulk solution. Taken together, these observations indicate that the substrates were progressively depleted as the reaction-diffusion front advanced through the PCM array from left to right. We also noticed that PCMs x_3y_1, x_4y_1 and x_3y_4, x_4y_4 always developed a higher mean fluorescence intensity compared to PCMs x_3y_2, x_4y_2 and x_3y_3, x_4y_3 (Supplementary Video 6, Supplementary Figure S9 blue and green plots). We attributed this to the PCMs in rows y_1 and y_4 being exposed to additional Glu diffusing along the top and the bottom of the field of view. By contrast, a control experiment carried out under diffusional equilibrium conditions where the enzymatically active 4x4 PCM array was placed on a PBS solution (10 mM, pH 6.8) preloaded with both Glc and *o*-PD (final concentrations 1.0 and 0.5 mM, respectively) showed a nearly immediate homogeneous fluorescence turn-on through the entire PCM array (Figure 4c, Supplementary Video 7, Supplementary Figure S10). The onset time of fluorescence of all 16 PCMs took place during the first 2-3 min of the experiment, and, as expected, it was independent of the spatial position of the PCMs (Figure 4d, Supplementary Table S3). The time-dependent mean fluorescence intensity curves reached a maximum after *ca.* 60 min, which was followed by a progressive decrease of the signal due to the depletion of the substrates and diffusion of the 2,3-DAP product into the bulk solution (Supplementary Figure S10). These observations are coherent with our initial hypothesis that 2D arrays of PCMs can be biochemically programmed to collectively detect and visualise advancing concentration gradients of substrates of interest under non-equilibrium conditions.

In order to improve our understanding of the transient spatiotemporal response of our enzymatically active 4x4 array of PCM, we performed additional experiments by single-component diffusion of either *o*-PD or Glc into a solution preloaded with the second substrate, *i.e.* Glc or *o*-PD, respectively. As was previously the case, in both experiments time-dependent mean fluorescence intensity analysis in general showed a propagating fluorescence wave that moved through the PCM array from left to right (Supplementary Videos 8, 9). This was associated with the progressive diffusion of the *o*-PD or Glc and with the HRP-mediated production of 2,3-DAP. However, by comparing these two different experiments, we also noticed some important differences in the spatiotemporal response of the 4x4 PCM array. When we preloaded Glu and diffused *o*-PD through the PCM array we observed a slow sequential fluorescence turn-on of columns x_1 and x_2, whereas columns x_3 and x_4 did not turn-on in the timeframe of the experiment (Supplementary Figure S11). Moreover, columns x_1 and x_2 continued to produce 2,3-DAP during the timeframe of the experiment and they did not turn-off as in the previous experiment where we diffused both Glc and *o*-PD. These observations are consistent with the rate limiting step of the overall process being the production of H_2O_2, rather than the diffusion of *o*-PD through the array. This was attributed to a low concentration of preloaded Glc in the system and to the slower catalytic reactivity of the GOx-containing protocells compared to those containing HRP. However, when we diffused Glc in a bulk solution pre-loaded in *o*-PD we observed a fast and sequential fluorescence turn-on of the entire array from columns x_1 to x_4, followed by a similarly fast and

sequential decrease of the fluorescence signal in each PCM (Supplementary Figure S12). We attributed this behaviour to a fast local production of H_2O_2 when the concentrated diffusion front of Glc reached the PCM columns, followed by a fast local depletion of *o*-PD substrate due to the high activity of the HRP-containing protocells that composed the PCMs. Since in this instance the rate limiting step of the overall process was the diffusion of Glc, this allowed for the estimation of a rate of diffusion for Glc of 9.1 ($\pm$ 0.2) x 10^{-8} m^2 s^{-1} under these experimental conditions (Supplementary Figure S13). In this experiment we also observed that PCMs x_3y_1, x_4y_1 and x_3y_4, x_4y_4 had a higher mean fluorescence intensity compared to PCMs x_3y_2, x_4y_2 and x_3y_3, x_4y_3 (Supplementary Video 9). This was consistent with what was observed in the previous experiment where we co-diffused Glc and *o*-PD and was due to the PCMs in rows y_1 and y_4 being exposed to additional Glu diffusing along the top and the bottom of the field of view.

Overall, these experiments showcase that PCM arrays provide a novel chemically programmable framework in which to systematically study information encoded in propagating reaction-diffusion gradients of chemicals, such as direction of the diffusing front, spatiotemporal changes in chemical concentrations, estimation of the diffusion rates of chemical species, and identification of rate-limiting steps of the PCM bioreactivity. These results therefore provide a first important example of spatially organised prototissues that can sense the external environment, trigger an endogenous coordinated response, and operate under non-equilibrium conditions, providing a new paradigm of prototissue engineering.

Conclusions

In summary, working towards fully autonomous synthetic tissues, we used bio-orthogonal chemistry for the programmed assembly of synthetic protocells into centimetre-sized tissue-like materials that are stable in water media, can communicate internally and with the external environment, and are capable of emergent non-equilibrium biochemical sensing. This was achieved by packing millions of bio-orthogonally reactive proteinosomes in oil at the water-air interface inside a PTFE mould floating on a 5 wt% solution of polysorbate 80. Robust PCMs were generated from the synergistic effect of surfactant-mediated oil removal, Marangoni flow, and interfacial bio-orthogonal ligation of the protocell building blocks.

We then showed that PCMs with complex 3D architectures could be easily constructed using this novel floating mould technique and we successfully generated patterns of different protocell phenotypes and stratified PCMs. It should now be possible to advance this methodology to generate even more complex 3D architectures where protocell populations with different specialised functions are patterned into individual layers of different thicknesses that can then be assembled into stratified PCMs. This would open up a way to the generation of PCMs with internalised complex communication pathways or to the microscale engineering of soft machines and devices that comprise localised components to carry out specific biosynthetic tasks.

The communication properties of the PCMs were then investigated by assembling PCMs capable of an internalised GOx/HRP enzyme cascade. These PCMs were capable of sensing the external environment and triggering a coordinated internalised biochemical response using an endogenously produced signalling molecule (H_2O_2). The unique communication properties of these materials were then employed to construct for the first time arrays of synthetic tissues that were capable of dynamically

extracting encoded information provided by the external environment in the form of unidirectional diffusing fronts of chemical species.

Our results open up a route from the synthetic construction of different protocell building blocks with adhesion capabilities to their programmed assembly and spatial integration into cm-sized tissue-like materials with precise architectures and geometries. These PCMs are stable in water and are capable of combining the specialisation of individual protocell types with the emergent spatiotemporal biochemical response of the ensemble. From a more general perspective, the programmed assembly of non-equilibrium materials capable of emerging bio-inspired functions from protocell building blocks addresses important challenges of bottom-up synthetic biology and biomimetic materials science and is expected to open new avenues towards novel organised platforms for tissue engineering, pharmacokinetics, personalised therapy, micro-bioreactor technologies, and soft robotics.

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Author contributions

All authors conceived the experiments, undertook data analysis, and discussed the results. P.G. devised the floating mould technique and coordinated the work. A.G., R.A., fabricated and characterised the protocellular materials. R.M. and S.D. performed SEM characterisation. S.C. developed the software to track the oil removal process and was involved in the analysis of fluorescence microscopy images. A.G. and P.G. devised and performed the non-equilibrium sensing experiments. All authors contributed to drafts of the manuscript. A.G. and P.G. wrote the final manuscript.

Competing interests

The authors declare no competing interests.