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Evolution of Self-Replicating DNA Towards Synthetic Life

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Some of the hallmark features of life as we know it is the ability to self-regenerate, self-replicate, and evolve. Substantial efforts in bottom-up synthetic biology generated a multitude of synthetic systems that reconstitute some of the processes found in living organisms. However, an integrated synthetic living system capable of self-regeneration, self-replication, and genome-level evolution has not yet been realized. My research program aims to address this challenge by an approach I call integrative evolution, which is based on Darwinian evolution of self-replicating gene networks in biomimetic compartments. As a first step towards this goal, we showed that *in vitro* transcription and translation-coupled self-amplifying DNA is capable of Darwinian evolution in cell-sized vesicles. Within ten rounds of evolution, we observed increased levels of overall self-amplification due to enrichment of adaptive mutations in the synthetic genome. To improve the level of the system's self-regeneration, my group will integrate additional functional modules in a way that couples their function to the overall amplification of the gene networks. This research program will contribute to improved understanding of fundamental nature of life and general processes that lead to the origins of life.

*Speaker

Combined Spectro-Electrochemical Analyses of Bio-Chemical Activities at Individual Artificial Cells

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The assembly, functioning and efficiency of the biomimetic artificial cells are studied by only few analytical techniques, mostly based on fluorescence measurements. Fluorescence techniques have major advantages for staining and imaging but also display limitations for quantitative assays. Then, there is potent interest for combining fluorescence with other spectroscopic techniques including electrochemistry to study synthetic cells. In this context, we developed approaches combining luminescence measurements with electrochemical detections of redox species released by Giant Unilamellar Vesicles (GUVs). Amperometric methods directly provide quantification of concentration variations of redox species and of their diffusion features. Thus, we performed combined measurements of H2O2 release by GUVs wherein glucose oxidase GOx activity was achieved. The amplex red assay image and measure by fluorescence the GOx activity (1) while a Platinum ultramicroelectrode directly detects at the liposome membrane the H2O2 flux following its internal production and membrane diffusion (2). Then, we developed an approach based on electrochemiluminescence (ECL) imaging of liposome permeabilization-release processes triggered by electroporation (3) or by antimicrobial peptides (4). ECL reagents -(Ru(bpy)3)2+ and tripropylamine - are encapsulated in GUVs during their formation. Then, liposomes are placed at the surface of a conductive and transparent ITO electrode, and when permeabilized their released content is electrochemically oxidized at ITO inducing light generation by ECL. This approach was also successfully transposed to monitor a NADH-based glucose dehydrogenase activity in GUVs (5). The simultaneous monitoring of ECL, photoluminescent and amperometric signals provide insightful, quantitative information on each liposome location, permeabilization and reactivity.

(1) P. Lefrançois et al., *Analyst* (2020), 145, 7922

(2) P. Lefrançois et al., *Analytical Chemistry* (2021), 93, 13143

(3) F. Ben Trad et al., *Analytical Chemistry* (2022), 94, 1686

(4) F. Ben Trad et al., *Chemical Biomedical Imaging* (2023), 1, 58
(5) F. Ben Trad et al. *Analytical Bioanalytical Chemistry* (2024), in press

*Speaker

Local environment in biomolecular condensates modulates enzymatic activity across length scales

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The mechanisms that underlie the regulation of enzymatic reactions by biomolecular condensates and their scaling with compartment size remain elusive. Here we use intrinsically disordered domains as building blocks to generate programmable enzymatic condensates of NADH-oxidase (NOX) with different sizes across length scales from nanometers to microns. These disordered domains are derived from three distinct RNA-binding proteins, each possessing different net charges, and result in the formation of condensates characterized by a comparable high local concentration of the enzyme yet within distinct environments. We show that solely the condensates exhibiting the highest recruitment of substrate and cofactor increase the enzymatic activity. Notably, the enzymatic rate is enhanced in condensates spanning a broad range of sizes, from nanometers to microns, indicating that emerging properties of condensates can appear already in assemblies in the nanometer size range. Furthermore, we observed a larger rate enhancement in smaller condensates. Our findings demonstrate that condensates can modulate enzymatic reactions by behaving as distinct effective solvent environments compared to the surrounding solution, paving the way for the design of novel protein-based heterogeneous biocatalysts.

*Speaker

A closed-loop optimization workflow for directed evolution of synthetic cells

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Building a living cell from separate components faces a major hurdle: the huge number of parameters that must be explored as the system's complexity increases. We address this challenge by combining automation and active learning algorithms to navigate the vast experimental parameter space. Our approach integrates (i) robotics for large-scale exploration of molecular contents (e.g., lipids and PURE system components), (ii) high-throughput screening of gene-expressing vesicles, and (iii) artificial intelligence to accelerate the searching of biochemical compositions that lead to improved or novel vesicle properties.
We developed a workflow for enhancing protein synthesis yield and kinetics using active learning (1) and Echo-assisted dispensing of 20 different PURE constituents. New compositions resulting in higher expression levels in bulk reactions have been discovered. Follow-up experiments aim at encapsulating optimized PURE inside liposomes to boost up the occurrence of phenotypes that are relevant to build a synthetic cell. This integrated approach will be applied to the expression and evolution of larger 'synthetic genomes'. Moreover, first steps towards a closed-loop optimization workflow will be established, whereby all key operational steps will be executed in an autonomous manner.
(1) Pandi *et al.* (2022) *Nat Commun* 13, 3876.

*Speaker

First step towards whole genome cloning of Bacillus subtilis in yeast by CReasPy-Fusion

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Genome engineering of microorganisms has become a standard in microbial biotechnology. In 2010, promising synthetic biology technologies using yeast as a platform for the assembly and engineering of synthetic bacterial genomes followed by their transplantation into a recipient cell have emerged. These technologies have led to the creation of the first synthetic cells and opened new avenues towards the construction of cells with fully controlled biological properties. The transfer of these advanced tools to microorganisms of interest such as the Gram+ bacterium *Bacillus subtilis* (*Bsu*), a biotechnology workhorse, will constitute a central advance, particularly in the improvement of bacterial strains for industrial purposes. As a first step towards that goal, an INRAE consortium set out to clone the entire *Bsu* genome in yeast using CReasPy-Fusion, a newly developed method that relies on direct fusion between bacterial protoplasts and yeast spheroplasts preloaded with a CRISPR-Cas9 tool. Efforts to date have demonstrated: (1) cell-to-cell DNA transfer between Gram+ *Bsu* bacteria and yeast cells, a phenomenon never described before; (2) the efficiency of a CRISPR-Cas9 system carried by yeast cells to capture and modify a shuttle plasmid during *Bsu*/yeast fusion; and last (3) the efficiency of the same CRISPR-Cas9 system to capture a 130-kb fragment of the *Bsu* genome, thus validating the CReasPy-Fusion method for cloning the *Bsu* genome. Since then, larger *Bsu* genome fragments were cloned in yeast; their capture appears to be facilitated by the addition beforehand of yeast ARS (autonomously replicating sequence) elements along the *Bsu* chromosome. With this knowledge, we now aim to clone the complete _˜3Mb genome of a genome-reduced *Bsu* strain in yeast.

*Speaker

Expanding the Toolbox for Engineering Functional Artificial Cell Compartments

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The bioinspired design of innovative soft materials that mimic key functions of living cells holds promise for developing novel solutions to major technological challenges in a range of industrial sectors. To realise this potential, a broad toolbox of functional components needs to be developed that can be integrated within the engineering design of these artificial cell mimics. We have developed a range of approaches to engineering the functionality of vesicle compartments. A major challenge in developing artificial cells for real world technologies is their requirement to maintain functionality over extended periods of time and across a wide range of environmental conditions. Lipid vesicles are not known for their durability. However polymer vesicles can overcome these shortcomings. That said, lipids are important bioactive constituents that facilitate the activity of integral proteins and recruit peripheral proteins. We have developed hybrid lipid-block copolymer vesicles, which enable formation of raft-like 2D compartments and support the function of integral proteins. These hybrid vesicles can enhance the functional lifetime of integral proteins by up to an order of magnitude and we have developed efficient, detergent-free methods for reconstitution.

Native cells are also highly compartmentalised and these compartment structures are necessarily dynamic to enable a wide range of critical cell functions. We have developed a range of native-like and synthetic approaches to achieving this in vesicle compartments. Natural ESCRT complexes remodel vesicle compartments with feedback-control via membrane tension and enable lipid sorting to form sub-compartments with liquid-ordered membranes. Synthetic nanoparticles can also mimic the membrane-sculpting functions of native proteins. Inorganic silica nanoparticles within a specific size range efficiently drive vesicle fusion and self-assembled cubosomes drive compartment growth and division.
Combining these tools alongside others developed within the wider community will enable the future engineering of highly functional cell-like particles with properties optimised to address our technology needs.

*Speaker

Development of Bioenergetic Hybrid Artificial Cells

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In eukaryotic cells, the organelle dedicated to energy conversion is the mitochondrion. In this context, we are developing hybrid artificial cells by encapsulating isolated mitochondria in giant synthetic liposomes and achieving bioenergetic activities within. Liposome unilamellar phospholipid membranes mimic natural cell membranes and thus provide a good structure to perform biological and biochemical reactions within a controlled micrometric environment.
The main source of energy for biological processes is the hydrolysis of ATP (adenosine triphosphate). Mitochondria are the main producers of cellular ATP, at least in aerobic processes.(1) In this study, we managed to encapsulate mitochondria (300-1300 nm size), isolated from *S. cerevisiae* yeasts, inside giant liposomes during their formation process. The inverted emulsion method using microfluidics offers a good success rate for the encapsulation of an initial solution containing mitochondria and selected substrates-reactants inside giant unilamellar vesicles (GUVs). Mitochondrial activity in GUVs is then studied by several methods including monitoring the mitochondrial membrane potential difference by epifluorescence microscopy with the fluorescent dye TMRM (tetramethylrhodamin-methylester), and measuring mitochondrial oxygen consumption using a Clark electrode. Their *in situ* activity is also modulated by the addition of respiratory chain substrates/inhibitors. (2) Enzymes can also be added to the liposome in combination with mitochondria, such as luciferase and horseradish peroxidase to detect by luminescence or fluorescence assays, respectively, the ATP and reactive oxygen species (ROS) production by mitochondria.(3)(4) The control of bioenergy production is a prerequisite to power other biomimetic processes in artificial cells in order at last to build up complex systems such as artificial organs.

(1) D.G Nicholls, S.J. Ferguson, *Elsevier* (2013), *Bioenergetics.*

(2) C. Colin et al., *Mitochondrial Medicine* (2021), 2276, 153-163.

(3) P. Lefrançois et al., *Anal. Chem.* (2021), 93, 13143-13151.
(4) P. Lefrançois et al., *Analyst* (2020), 145, 7922-7931.

*Speaker

Ghost in the cell: artificial cells via enzyme-mediated polymer synthesis and self-assembly

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Artificial cells, serving as biomimetic microstructures, emulate the functionalities of natural cells, becoming building blocks in molecular systems engineering and serving as vessels for synthetic biology. We unveil the creation of polymer-based artificial cells, synthesized enzymatically, with a capability for protein expression. The construction of artificial cells was accomplished utilizing biocatalytic atom transfer radical polymerization-induced self-assembly (bioPISA). To this end, myoglobin facilitates the synthesis of amphiphilic block copolymers, which self-organize into various structures including micelles, worm-like micelles, polymersomes, and giant unilamellar vesicles (GUVs). Throughout the polymerization process, the GUVs encapsulate diverse cargo, encompassing enzymes, nanoparticles, microparticles, plasmids, and even cell lysate. Consequently, the formulated artificial cells function as microreactors, facilitating enzymatic reactions and osteoblast-inspired biomineralization. Furthermore, upon being supplied with amino acids, they are able to express proteins, including a fluorescent protein and actin. Actin polymerizes within the vesicles, modifying the internal structure of the artificial cells by forming a cytoskeleton mimic. Therefore, GUVs produced via bioPISA can emulate bacteria, constituting a microscopic reaction compartment that holds genetic information, enabling protein expression upon induction. Moreover, artificial cells can be further equipped with internal compartments in sequential reactions, imitating the eukaryotic cell subdivisions. Belluati, A., Jimaja, S., Chadwick, R.J. *et al.* Artificial cell synthesis using biocatalytic polymerization-induced self-assembly. *Nat. Chem.* (2023). https://doi.org/10.1038/s41557-023-01391-y

*Speaker

Creating temporal resolution for in vitro protein synthesis in artificial cells

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Using membrane compartments as vessels for artificial cells creates limitations for controlling the concentrations of components after the system has been initialized. *In vitro* protein synthesis allows to initiate the production of protein in the system, but remains poorly controllable over time. Transcription repressors can be useful tools to provide controllable dynamics of gene expression in the artificial cell system. In this work, we use giant unilamellar vesicles with encapsulated PURE *in vitro* protein synthesis system. We demonstrate that gene expression can be repressed by TetR and derepressed by addition of anhydrotetracycline. We show that functional proteins can be regulated using the MinCDE protein system as an example. MinCDE proteins are involved in bacterial division, they create pole-to-pole oscillations that position the division ring in the middle of the cell. They have also been proven a robust tool for membrane patterning and spatial positioning of membrane binders *in vitro,* in particular for positioning protein filament bundles and contractile rings. Therefore, being able to regulate MinCDE expression and oscillation in GUVs brings us one step closer to spatiotemporally controlled artificial cell division.

*Speaker

Synthetic metabolic BZ hydrogel actuator

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The existence of living organisms is evidence that functional motile autonomous materials can be built robustly using macromolecules. However, the challenge of building from the molecule to the macroscopic scale a purely synthetic functional chemomechanical material that harness chemical energy to produce motion is unmet. This scientific effort could lead to the creation of life-like machines with augmented performance compared to what evolution has created. Inspired by Yoshida's work, we create synthetic autonomous chemomechanical polymeric materials made of acrylamide hydrogels doped with the catalyst of the Belousov Zhabotinsky(BZ) reaction . Our two-step synthesis technique allows independent optimization of the geometry, the chemical, and the mechanical properties of BZ gels. We identify the role of the surrounding medium chemistry and gel radius for the occurrence of BZ gel oscillations, quantified by the Damkohler number, ratio of chemical reaction to diffusion rates. Tuning the BZ gel size to maximize its chemomechanical oscillation amplitude, we find that its oscillatory strain amplitude is limited by the timescale of gel swelling relative to the chemical oscillation period. We also demonstrate that hydrogel-based actuators with an internal chemical clock develop stresses as high as 30kPa, which is only a factor of 3 less than muscle tissue. Through fitting our experimental data with a Flory-Huggins theory for polyelectrolytes, we argue that the gel chemomechanics arises from the change in solvation energy. These findings contribute to the understanding of Nature's ability to harvest chemical energy for actuation.

*Speaker

Engineering differential transport through N-terminal linker functionalization of self-inserting nanopores

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Despite significant advancements, engineering-controlled transport across synthetic membranes presents a persistent challenge. Integration of proteins into synthetic membranes typically requires complex biological machinery, displays a strong dependence on membrane composition, or relies on detergents. To overcome these challenges, our work leverages the properties of alpha-Hemolysin, a well-characterized self-inserting nanopore.
We have introduced a new small molecule differential transport property by strategically customizing the pore entrance. By combining confocal microscopy with electrophysiological measurements, we provide a robust validation of successful pore integration and functionality for all engineered pore variants. Ongoing assessment using plate reader assays as well as microfluidics reveals the differential transport between pore variants for small, charged molecule dyes. A number of different applications may be enabled with this pore customization strategy, with only small and straightforward modifications. For example, we are designing in-vitro biochemical pathways that are selectively separated by a functionalized membrane with engineered self-inserting pores.
Selective transport of vital metabolites, such as ATP, could pave the way for spatially separated bioreactions, intricate synthetic cell systems, and functionalized biomaterials. This customization strategy is a simple and significant step towards achieving controlled transport, opening doors for spatially controlled reactions.

*Speaker

Active Droplets: Droplets Regulated by Chemical Reaction Cycles

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Active droplets are droplets regulated by chemical reaction cycles. Such droplets are common in our cells as membraneless organelles, *i.e.*, organelles that rely on phase separation of RNA and proteins from the cytosol, frequently regulated through reaction cycles (1). Due to their non-equilibrium nature, these droplets are endowed with properties we do not observe for in-equilibrium self-assembly. For example, theoretical physicists have predicted they can avoid being subject to Ostwald ripening (2), while another study predicted they can spontaneously self-divide (3). Their relevance in biology and exciting new properties are good reasons to build them from the bottom-up.
Therefore, we developed a chemical reaction cycle that continuously activates and deactivates molecules for phase separation at the expense of chemical fuel (4,5). In response to chemical fuel, droplets emerge that compete for scarce resources. Each droplet receives molecules from the outside phase, where activation happens. That droplet loses material due to the deactivation reaction. That interplay of activation outside and deactivation inside results in fascinating behavior; behavior like droplet emergence, decay, and self-division. In this lecture, I will present the highlights of our findings, offer design rules for active droplets, and give an outlook on their role in the synthesis of life.

(1) Shin, Y, et al. *Science* **2017**, *357* (6357), eaaf4382.

(2) Zwicker, D., *Physical Review E* **2015**, *92* (1).

(3) Zwicker, D. *Nature Physics* **2017**, *13* (4), 408

(4) Donau, C., *Nature Commun* **2020**, *11* (1), 5167.

(5) Späth, F.; *J. Am. Chem. Soc.* **2021**, *143* (12), 4782

*Speaker

Counting mRNAs: single-molecule in vitro transcription (smIVT) assay

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Transcription is a vital cellular process in which RNA polymerase produces messenger RNAs (mRNA) from a DNA template. On its trajectory toward a viable artificial cell, synthetic biology aims to reliably reproduce transcription in a confined environment and thoroughly understand how it works. To this end, a number of commercially-available/lab-made transcription systems have been established for in vitro applications. Moreover, using fluorescent probes, researchers could follow the changes in RNA concentration over time inside giant unilamellar vesicles (GUVs). However, most of these measurements have DNA concentrations far exceeding those of natural cells, where there is usually only a single DNA copy. Here, we show a single-molecule in vitro transcription (smIVT) assay that goes beyond these limitations and allows us to track single DNA and RNA molecules in solution and inside GUVs. We have achieved this by employing a molecular beacon (MB), a fluorescent probe that becomes more fluorescently active after interacting with the transcript mRNA. The molecular beacon we used is a 26-base long DNA hairpin whose ends were modified with ATTO647N dye and an Iowa Black RQ quencher. The dye is efficiently quenched while the beacon stays closed and diffuses in the solution. However, the dye fluoresces as soon as the binding to the complementary sequence on the mRNA takes place. The template DNA we used here had 32 MB-complementary sequence repeats to ensure a signal-to-noise ratio sufficient for detecting single mRNAs. Based on the results obtained using our single-molecule transcription assay, we present here (i) a ranking of different commercially available in vitro transcription kits; (ii) dependencies of transcription yields on temperature, time and template concentration; (iii) a quantitative description of GUV-confined transcription. Thus, we establish smIVT assay as a remarkable technique to scrutinize in vitro transcription with single-molecule resolution.

*Speaker

Self-Regulated Metabolic and Genetic Linked in Vitro Network

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Cellular resource allocation and efficient utilization are paramount for the survival and functionality of living organisms. Central to this process is the regulation of gene expression in response to key metabolites, a fundamental strategy employed by the living cells. In this study we build an *in vitro* self-regulated metabolic and genetic linked network (MGLN) able to perform "decision-making" *i.e.*, it autonomously activates its genetic layer upon producing a target metabolite. We achieved this goal by integrating the metabolism of the crotonyl-CoA/ethylmalonyl-CoA/hydroxy butyryl-CoA (CETCH) cycle producing glycolate, with cell-free protein synthesis using recombinant elements (PURE). We demonstrate this concept by regulating gene expression with glycolate produced from CO2, and glycolate-inducible transcription factor GlcR from *Paracoccus denitrificans*. We are currently further optimizing such MGLN by using a machine-learning based algorithm (METIS), recently developed in our lab, for bottom-up assembling cell-free systems able to autonomously self-repair.

*Speaker

Light- and magnetism-controlled synthetic cells for biology and medicine

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Synthetic cells, lipid membrane-bound compartments that mimic living cells, are a promising technology for studying living systems and as drug delivery vehicles. However, real-world application of synthetic cells requires methods of controlling their function for spatiotemporal activation and to reduce off-target effects. To address this, we are developing methods to externally control these soft biomaterials with various biologically- and medically-applicable stimuli, by chemically modifying the nucleic acid component. We are then using our synthetic cells to mimic living processes and form controllable interfaces between living and non-living materials. Previously, we have developed the first dual light-activated AND gate within synthetic cells and light-activated synthetic cells that can communicate with bacteria. To enable activation deep inside the body, we have now developed synthetic cells that can be controlled with a magnetic field. In the future, our controllable synthetic cells may form the basis of targeted therapeutics and new technologies for basic research.

*Speaker

Impact of NusG-paralog RfaH on transcribing bacterial RNA polymerase

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Transcription and translation machineries can be coupled in bacterial systems, with specific factors bridging the RNA polymerase (RNAP) and the trailing ribosome. Among these, RfaH protein is a NusG paralog that is involved in the expression of genes obtained through horizontal transfer and has been proposed to couple the RNAP and the ribosome. To this end, the presence of an operon pausing site (*ops*) is required, at which the RNAP backtracks after recruitment of RfaH and hyper-stabilization of the pause.
We developed a high-throughput magnetic tweezers assay to investigate how RfaH impacts the transcribing *E. coli* RNAP at the single-molecule level. We show that the increase in temperature only mildly affects the RNAP elongation dynamics, and effect further mitigated by the presence of spermidine in the reaction solution. On the other hand, decreasing the NTP concentration significantly increases the pausing probability. We also characterized the pausing dynamics of RNAP on the *ops* site as a function of the aforementioned conditions.

Based on this acquired knowledge, it was possible to establish whether and how the presence of RfaH can influence the behaviour of the RNAP at the *ops* site. Specifically, we confirm that RfaH recruitment increases the duration of the pause, independently from the conditions it was tested in. We show that adding GreA in the reaction solution decreases the duration of the pause and consequently increases the percentage of RNAP escaping the pause during the experiment. These results therefore support the backtracking origin of the RfaH pause.
Our work paves the way towards a mechanistic understanding of RfaH recruitment, the impact of RfaH on transcription and establish the foundation to investigate transcription-translation coupling at the single-molecule level.

*Speaker

Synthetic cell-based 3D artificial lymph nodes to study the activation dynamics of T-cells

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Ex vivo T-cell activation and expansion is crucial for effective immunotherapy. In this context, synthetic tissue-mimics, acting as artificial antigen presenting cells (aAPCs) and in vitro lymphoid tissue replicas, are a promising approach. However, mimicking the biochemical and biophysical cues provided by natural APCs and required for reliable expansion of therapeutic relevant T-cell phenotypes is a formidable engineering challenge. For strong T-cell activation, established and commercially available T-cell activation beads relay on biochemical triggers in the form of immune-stimulatory antibodies present on their non-mobile surface.

In contrast to this, I demonstrate that lateral ligand mobility as a key factor involved in T-cell activation and expansion. For this, I developed a synthetic bottom-up cell assembly strategy to form dispersed droplet-supported lipid bilayers (dsLBs) able to emulate the natural lipid membrane characteristic of APCs. To take the system one step closer to in vivo conditions, I engineered the dispersed synthetic cells to self-assemble into 3-dimensional reaction rooms to form artificial lymphoid bottom-up tissue (lymphBUTs) structures. LymphBUTs provide T-cells with controllable biochemical and biophysical cues for a reliable therapeutically relevant T-cell expansion. Co-culture of T-cells within lymphBUTs result in infiltration of the T-cells into the synthetic tissue and subsequent expansion of a CD8+ phenotype with regulatory- and memory-like function.

In a future perspective, the focus will be on engineering heterogeneous lymphBUTs comprising functional reaction zones. This compartmentalization within the lymphBUTs leads to functional heterogeneity which offers zones for separate immune reactions. In a broader point of view, self-assembled BUT structures with therapeutic potential opens doors for many applications in synthetic biology.

*Speaker

How does the oil influence the formation of GUVs by the droplet transfer method?

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The droplet transfer method has been an essential tool for the generation of Giant Unilamellar Vesicles (GUVs) over the past two decades, playing a crucial role in the construction of artificial cells. Despite its widespread adoption, a comprehensive investigation of the factors affecting this protocol has been lacking. Among these factors, the oil phase, utilized to dissolve phospholipids, has emerged as critical.
A wide range of oils has been explored previously, but an extensive characterization of these is not present in current literature. This study addresses this knowledge gap, attempting to elucidate the interplay between the physical and chemical properties of the oil phase and their effects on GUV formation by the droplet transfer method. Two main aspects may influence the protocol: a direct contribution of oil molecules to GUV membranes and the solubility of phospholipids in the oil phase. Hydrophobic molecules within the oil phase can potentially integrate into the phospholipid bilayer, altering the physicochemical properties of GUV membranes. Enhanced solubility of phospholipids in the oil phase may promote greater freedom of movement, potentially influencing both the equilibrium at the water/oil interface in the emulsion and the formation of a second phospholipid layer during the droplet transfer into the aqueous phase.
To assess the impact of various oil phases on GUV formation using the droplet transfer method, we systematically investigate oil phases with diverse physicochemical properties. These are exploited to dissolve dioleoylphosphatidylcholine (DOPC), a phospholipid commonly used to fabricate GUVs. The analysis includes interfacial tension between oil and water phases, Z-potential, and dimensional distribution of GUVs. The outcomes of this research are aimed at improving our understanding of the droplet transfer method and at providing a set of procedures for dedicated optimizations, including derivative methods like cDICE, droplet shooting, and microfluidics, potentially boosting the research in artificial cells.

*Speaker

Regulating condensates within synthetic cells via segregative phase separation

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Living cells are capable of performing a myriad of biological reactions owing to the intricate intracellular compartmentalization. Along with membrane-bound organelles, many cellular compartments are membraneless condensates, formed via associative phase separation (APS), resulting in biopolymer-rich dense liquid phases. Understanding and applying APS is crucial for engineering synthetic cell systems and is actively being explored for diverse applications. However, optimal spatiotemporal control over condensates, particularly preserving their identity and location within vesicles or at the membrane, has remained challenging. In this study, we employ segregative phase separation (SPS, resulting in co-existing but segregated polymer-rich phases), within synthetic vesicles to gain spatiotemporal control over the isolation and localization of condensates. Using OLA, an on-chip liposome production technique, we encapsulate the model components for both the phase separation systems – polyethylene glycol/dextran for SPS and polylysine/ATP for APS – in liposomes. We trigger SPS using hyperosmotic shock to increase the concentrations, and trigger APS using pH change to modulate charge-based interactions, leading to a cascade of self-regulated events. First, the dextran-rich domains wet the membrane, transforming the liposomes into flower-shaped morphologies. Remarkably, strong partitioning of polylysine into the dextran-rich phase leads to condensate formation strictly in these membrane-bound micro-domains. The isolated coacervates further interact with the membrane to form bud-like structures. Thus, SPS provides multiple chambers within a liposome to nucleate and further isolate the condensates. We also observe a significant reduction in the diffusion of confined condensates, owing to the increased viscosity and membrane interactions. In conclusion, we propose SPS as an effective condensate regulation strategy in synthetic cells by demonstrating i) molecular enrichment and promoting coacervation within specific domains, ii) isolation and restrictive diffusion of condensates, and iii) tuneable interactions with the lipid membrane. We believe our approach could be of great potential for engineering membraneless organelles in synthetic cells.

*Speaker

Growing and switching artificial cytoskeletons in the viscoelastic confinement of DNA synthetic cells

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Vital intracellular structures, such as membraneless organelles and cytoskeletons, form in the crowded cytoplasm with defined viscoelastic properties. While the influence of simple crowding has been investigated for catalysis and self-assembly, it is still largely unclear how the viscoelastic properties of a matrix regulate structure formation in compartmentalized systems. Here, we build all-DNA synthetic cells with a high degree of crowding and controllable viscoelasticity to investigate their effects on the formation of artificial cytoskeletons in confinement. Using a specific co-assembly approach for the fabrication of synthetic cells, we introduce up to three different DNA barcodes with adjustable concentrations in the core of the synthetic cells. These barcodes allow selective enrichment of DNA building blocks at desired concentrations to assemble into artificial fibrillar cytoskeletons with different morphology. We find mechanistic differences to typical solution assembly and show a significant influence of viscoelastic confinement on assembly. Furthermore, we introduce light and molecular stimuli to control structure formation and show how orthogonally barcoded DNA synthetic cells can selectively recruit their cytoskeletal precursors in a competitive species pool, which leads to self-sorted artificial cytoskeletons formation in various populations of synthetic cells. The combination of controlled enrichment using DNA barcodes and high tunability of the viscoelastic properties of the compartments provides a versatile synthetic cell platform to fundamentally study self-assembly under viscoelastic confinement and guide the design of functional synthetic cells that can generate functions via embedded secondary structures.

*Speaker

De novo design and assembly of minimal chromosomes for the synthetic cell

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A major challenge for the bottom-up construction of a minimal synthetic cell is *de novo* design and assembly of a DNA genome encoding all cellular functions. Previous research in our lab has established DNA expression using PURE system in liposomes as a suitable chassis. However, only relatively short coding DNA templates have so far been designed and expressed inside liposomes, restricting the number of encoded functions. In this study, we have designed three synthetic genomes of increasing size (39, 96 and 103 kbp) and number of genes (15, 45 and 47) encoding (parts of) the modules for phospholipid biosynthesis, DNA replication, cell division and translation. Each design includes fluorescent reporters for PURE expression and origins of replication compatible with the protein-primed Phi29 DNA replication system. A suitable method for the assembly of such large DNA from multiple fragments (up to 18) is homologous recombination in the yeast *Saccharomyces cerevisiae*. We have identified two challenges in the compatibility of this method with expression in PURE system: (1) assembly of sequences with repeats arising from the limited choice of regulatory elements in PURE and (2) isolation of the assembled synthetic chromosome from yeast, which results in DNA with low concentration and purity. To streamline the assembly-to-characterisation pipeline, we incorporated marker fragments between each coding fragment for easy screening of correct assemblies, and included a BAC backbone to enable shuttling to *E. coli* for amplification and isolation with high DNA yield. After sequence verification and isolation of correct assemblies, the chromosomes will be tested as template for PURE expression and Phi29 DNA replication. Future efforts will focus on engineering these synthetic chromosomes to create genetic diversity, with the aim of module integration through *in vitro* evolution campaigns in liposomes.

*Speaker

ATP recyling in giant-unilamellar vesicles via the breakdown of arginine

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Many processes in a living cell rely on ATP as an energy source1. We present a simple system to generate ATP via the breakdown of arginine to ornithine plus ammonia and CO2. Arginine is imported into the vesicles by the arginine/ornithine antiporter ArcD. The arginine is then deaminated to citrulline, which in turn is converted to ornithine plus the high-energy intermediate carbamoyl-phosphate. Ornithine is exported by ArcD in exchange for arginine, which prevents the build-up of reaction products inside the vesicles. The carbamoyl-phosphate drives the phosphorylation of ADP to ATP by a kinase. This system remains out-of-equilibrium, provided the ATP is consumed by another process such as the synthesis of precursors for membrane growth or volume regulation of the vesicles2,3. The arginine breakdown pathway has been extensively studied and used in submicron-size vesicles, and we now present its usefulness in giant-unilamellar vesicles (GUVs) and the study of ATP-dependent processes at the single vesicle level4. The performance of the ATP-producing pathway is assessed by fluorescence-based sensors that are encapsulated in the GUVs. We present on the formation of the vesicles by gel-assisted swelling, the trapping and analysis of the vesicles in microfluidic devices, and the membrane permeation of arginine and pathway intermediates.

1: Bailoni, E. *et al.* Minimal Out-of-Equilibrium Metabolism for Synthetic Cells: A Membrane Perspective. *ACS Synth. Biol.* **12**, 922–946 (2023).

2: Pols, T. *et al.* A synthetic metabolic network for physicochemical homeostasis. *Nat Commun* **10**, 4239 (2019).

3: Poolman, B. *et al. Synthetic Organelles for Energy Conservation and Delivery of Building Blocks for Lipid Biosynthesis.* https://www.researchsquare.com/article/rs-3385355/v1 (2023) doi:10.21203/rs.3.rs-3385355/v1.

4: Coenradij, J. *et al.* Construction of Out-of-equilibrium Metabolic Networks in Nano- and Micrometer-sized Vesicles. JoVE. *In press*

*Speaker

Transcription and In Vitro selection inside lipid vesicles to identify cooperating ribozymes

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In vitro selection simulates natural evolutionary processes, including the optimization of enzymes and ribozymes during the emergence of life. Typically, in vitro selection experiments identify individual oligomers with important functions. However, cooperative interactions between oligomers (e.g. polypeptide subunits of a multimeric polymerase) enable unique activities that modern cells rely on. To select for cooperative interactions, biopolymers must be encapsulated inside compartments. Water-in-oil emulsions are convenient to compartmentalize ribozymes and their substrates, but cells are bounded by lipid membranes. By developing methods for in vitro selection experiments in lipid vesicles, we aim to simulate early evolutionary processes. Expression and selection of ribozymes inside lipid vesicles is challenging because vesicle formation is hindered by moderate concentrations of divalent cations, which are needed to support transcription. We are developing a novel strategy for in vitro transcription inside lipid vesicles by optimizing lipid composition and MgCl2 concentration. We have identified conditions compatible with both expression of active ligase ribozymes and stability of lipid vesicles. We will present our progress applying this strategy towards in vitro selection of multimeric ligase ribozymes from random sequences. Our platform provides a powerful way to screen catalysts that may provide insight into the emergence of life on Earth.

*Speaker

Bottom-Up Approaches to Designing Dynamic Behaviors in Artificial Cells

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My research group focuses on the development of artificial constructs that simulate the structure and functionality of biological cells, using a combination of synthetic and hybrid molecular frameworks engineered for precise manipulation. By emulating the fundamental characteristics and behaviours of living cells, these cell-like systems offer insights into biological systems, paving the way for a variety of functional applications. A notable challenge in the field of bottom-up synthetic biology is the creation of these synthetic entities capable of dynamic behaviours, such as fusion, material uptake, and autonomous, directional movement in response to environmental stimuli, reflecting the intricate processes of biological communication and organization. The endeavour to construct life-like systems that are both manipulable and interpretable advances our comprehension of life's origins and drives scientific discovery. The fabrication of custom-designed, dynamic cell-like systems holds the potential for extensive application in clinical and industrial settings.
This rapidly advancing field promises to revolutionize the landscape with the introduction of artificial cell devices powered by biological compounds. These artificial cell systems are poised to make significant contributions, from aiding in bioremediation efforts to facilitating the creation of biomimetic tissues and materials with controlled spatiotemporal self-organization, showcasing the potential for applications in both environmental and biomedical engineering.

*Speaker

Artificial cells with reconfigurable contents and viscosity

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[4] Department of Chemical Engineering [Imperial College London] – United Kingdom

Cellular processes are driven by communication between cell and environment, and biophysical rearrangements such as the phase behaviours of lipids and biomolecular condensates. In mimicking the behaviours of living cells, artificial cells can take advantage of both established biological pathways, and novel synthetic processes. Herein two examples of the latter are demonstrated, that offer alternatives in light- and heat-triggered synthetic cell communication, and content exchange without the use of membrane proteins. Additionally, dynamic control over artificial cell viscosity and size is demonstrated, that could offer utility in the regulation of biomolecular reactions such as transcription and translation.
It is demonstrated that optical heating of nanoparticles is capable of permeabilising thermoresponsive SUVs, allowing the engineering of photo- and thermo-responsive artificial cells. The encapsulation and triggered release of fluorescence pre-cursors inside these systems offers a simple model of inorganically induced synthetic cell communication, with enzymatic conversion of the released moieties offering a real-time fluorescence readout. This offers a novel pathway in synthetic cell communication, with the potential for integration with biological systems without cross-talk.

Moreover, to date synthetic cells have typically compartmentalised either fluids or gels. In order to better mimic the dynamic mechano-biological properties of cells, we have designed synthetic cells that are able to responsively alter their own size and internal microenvironment. In particular, osmolarity-induced growth and shrinkage, and the use of redox-responsive crosslinks to trigger changes in internal viscosity between a liquid and gel state. Switching between these states provides control over the mechanophysical properties and permeabilization of synthetic cells. Such switching behaviour has broad implications, including in membrane protein activation, and control over reaction rates and content release.
The successful integration of these technologies with complementary behaviours offers significant potential in developing an autonomous artificial cell capable of sensing and responding to its environment in increasingly dynamical ways.

*Speaker

Transcription-Translation of E.coli modified genome in synthetic cells

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CoSyCell project aims at developing hybrid compartments made of all-in-water emulsion droplets covered by a lipid membrane. The all-in-water emulsion droplets will favour spontaneous sequestration of DNA, proteins and other biological molecules, while the lipid membrane at the surface of the droplets will ensure long-term encapsulation of these molecules within the compartments. We chose to work with biological material from *E.coli*, strain BL21. We first extracted the transcription-translation machinery (TX-TL) from bacteria. On the other hand, we wanted to modify the genome of the same strain, carried out by inserting mCherry and gfp gene at two different places in the genome with CRIPR-Cas9. The final objective is to use modified genome and TX-TL as aqueous phase in the synthetic cell and see if the system is able to produce proteins. Fluorescence measurement could testify to the completion of transcription and translation of the modified genome, by the TX-TL machinery extracted from the bacteria. Droplet transfer method or microfluidic are two different method that can be considered to create synthetic cells. The 1st technic is less controlled because the droplets size will not be homogenous. The 2nd technic will allow to obtain droplets of the same size.

*Speaker

Breaking bacterial genome in modules

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To create a synthetic cell, it will be necessary to include all the genetic information for the basic functions of life into a synthetic genome. When such a genome is generated bottom-up, a modular design seems the most plausible strategy. One module could encode all genes related to a defined function: e.g. transcription, translation, chromosome replication, cell division, central metabolism, etc. In natural genomes, genes for many functions are scattered and the expression of genes from natural genomes appears to be affected by features like position, orientation and context. For designing a synthetic modular genome new design rules have to be developed, tested and optimized. Initially, we will restructure parts of the natural *E. coli* genome into modules and test the functionality of different designs. We will modularize genes related to the central metabolism, which are typically scattered in the natural genome. We will use different modular design strategies and test those for cellular fitness and by transcriptome and proteome analyses. This will lead to a gain of insight about the possibilities and rules for synthetic genome design.

*Speaker

Optimising in vitro transcription for use in synthetic cells

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Synthetic cells functionalised with an *in vitro* transcription (IVT) system offer significant potential in nanotechnology and therapeutics. The IVT system would enable *in situ* synthesis of RNA aptamers for biosensing and short interfering RNA or messenger RNA to control neighbouring cells. However, commercial IVT kits are not cost-effective, have fixed concentrations of components, and may contain further undisclosed components. This makes them difficult to customise for many applications and balance the internal and external solution of a synthetic cell. Furthermore, the commercial kit buffers possess a high Mg^2 concentration, which causes synthetic cells to aggregate. To address these issues, we have studied the dependence of IVT on each component and generated an optimised IVT system for use in synthetic cells. The commercial kit buffer was simplified into fewer components, helping to balance osmolarity and prevent component leakage. The Mg^2 and NTP concentrations were also optimised to maximise IVT yield, while minimising synthetic cell aggregation. This optimised IVT buffer was encapsulated into synthetic cells, with functional *in situ* expression after encapsulation. The cost-effective and synthetic cell-optimised IVT system can easily be modified to introduce controllability through the encapsulation of light-activated DNA. This optimised system will help to unlock the full potential of synthetic cells for real-world applications, including in medicine and biotechnology.

*Speaker

Stereoselective, nonenzymatic aminoacylation gives insight to the origins of homochiral life

Joshua Davisson * [1], Evan Kalb [1], Isaac Knudson [2], Aaron Engelhart [1], Alanna Schepartz [2], Katarzyna Adamala [1]

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Life as we know it depends on the homochirality of nucleic acids and proteins. However, there is no widely accepted explanation for why life uses only D-RNA and L-amino acids. Here we demonstrate a prebiotically plausible method of nonenzymatic aminoacylation in a water ice-eutectic phase. These reactions use prebiotically plausible substrates and conditions, producing high yields of aminoacyl-tRNAs that are active in translation. Surprisingly, we discovered these nonenzymatic aminoacylation conditions were generally stereoselective, favoring coupling of amino acids and RNA of opposite configurations. Biologically relevant D-RNA shows greater aminoacylation yields for L over D-amino acids. The opposite was true for the mirror image polymers, L-RNA resulted in greater yields with D over L-amino acids. Stereoselectivity of aminoacylation being dependent on nucleic acid chirality highlights a link between amino acid and nucleic acid configuration that is still present in modern biomolecules. A preference for D-RNA and L-amino acids provides insight into the chirality of the RNA world, and may explain proteins being exclusively composed of L-α-amino acids today.

*Speaker

Translation of non-natural 2'-fluoro mRNAs in cell-free protein expression system

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Biological systems rely on the accurate decoding of mRNA molecules into functional proteins. Ribosomes have evolved the ability to distinguish cognate from near-cognate tRNAs, independently of the codon-anticodon pair present in the A site. Specifically, the universally conserved 16S rRNA bases G530, A1492 and A1493 form hydrogen bonds with the 2'-hydroxyl group in the mRNA backbone in the mRNA-tRNA complex, selecting for a specific geometry present in cognate codon-anticodon helices. Near-cognate tRNAs produce a distinct codon-anticodon helix geometry that disrupts hydrogen bond formation, thus preventing accurate mRNA decoding. By checking for both hydrogen bond formation and correct codon-anticodon geometry, this process restricts accurate translation of non-natural transcripts by wild-type ribosomes. To test the limitations of this system, we expressed non-natural mRNAs with 2'-fluoro modified backbones, which disrupt hydrogen bond formation, but preserve the codon-anticodon helix steric positioning with respect to the conserved bases in the decoding cleft. We used the PURE cell-free protein expression system to test the translation of partially or fully fluorinated sfGFP mRNAs and detected fluorescence with mRNAs containing either 2'F guanosine or 2'F cytidine. To investigate whether specific nucleotide combinations or codon preferences affect the translation accuracy of fluorinated transcripts, we used a split GFP reporter encoded with different combinations of 2'F-modified nucleotides. Given the limitations of wild-type ribosomes, directed evolution of ribosomes in cell-free systems could enable translation of non-natural transcripts. To this end, we have built a library of the 16S rRNA between h44 and h45, with which we aim to evolve a new version of the 16S rRNA decoding cleft that is capable of accurately decoding 2'F mRNA.

*Speaker

Dynamin A as a one-component division machinery for synthetic cells

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Reconstituting cell division from the bottom up is one of the fundamental challenges in our quest to build a fully autonomous synthetic cell. In this work, we achieve reconstitution of the bacterial protein Dynamin A inside dumbbell-shaped liposomes, mimicking the shape of a dividing cell (1). We show that Dynamin A spontaneously assembles at the inner leaflet of the neck of dumbbell liposomes and efficiently induces both membrane hemi-scission and full scission (2). We unambiguously demonstrate such membrane rearrangement events using Fluorescence Recovery After Photobleaching experiments on a large dataset of dumbbells. Thus, we propose Dynamin A as a simple, one-component system that is compatible with *in vitro* transcription-translation systems and that represents an attractive candidate for building a division machinery for synthetic cells. Implementation of cell division within a synthetic cell cycle will also be discussed.
(1) De Franceschi et al. Synthetic Membrane Shaper for Controlled Liposome Deformation. ACS Nano. 2022 Nov 28;17(2):966–78.
(2) De Franceschi et al. Dynamin A as a one-component division machinery for synthetic cells. Nat Nanotechnology. 2024 Jan;19(1):70-76.

*Speaker

Ultra-deformable cell-inspired GUV-based microrobots infiltrating narrow spaces

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Microscale robotics opens the possibility to design untethered medical devices that can reach remote areas of the human body otherwise inaccessible. One of the biggest challenges in navigating through the body's biological fluids and tissues is represented by the complex and restrictive environment created by cells and networks of molecules, with interstices often significantly smaller than the characteristic size of microrobots.
This same challenge is faced by the cells of our immune system. Their strategy to traverse vessels and tissues through narrow interstices is based on a continuous adaptation to the surrounding environment through large body deformations. We take inspiration from leukocytes to design and fabricate ultra-deformable microrobots consisting of phospholipid-based giant unilamellar vesicles (GUVs) encapsulating a liquid magnetic core. This structure mimics the physical properties of natural cells, endowing the devices with a passive compliance that allows them to squeeze and deform in response to obstacles and constrictive spaces.

We fabricate ferrofluid-loaded GUVs through the droplet transfer method. We then test their capability to infiltrate narrow spaces by magnetically pulling them through channels that exert upon them a gradually more forceful confinement. We verify that the microrobots can infiltrate spaces smaller than their characteristic size and sustain significant body deformations without breakage. We then observe that the interplay of magnetic actuation and confinement can induce different behaviours in terms of deformation and velocity, deviating significantly from the behaviour of microrobots in free space.
We aim to further enhance the compliant properties of the microrobots by analysing and optimising the lipidic composition of the membranes; furthermore, we aim to assess the effect of osmotic deflation on the infiltration and deformation capability. This can ultimately lead to the realisation of cell-inspired, GUV-based microrobots capable of traversing unknown complex environments by squeezing through narrow spaces and bypassing obstacles.

*Speaker

Antibody epitope characterization in a multiplex surface-based cell-free expression platform

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Antigen antibody recognition is at the heart of the immune system's capacity to recognize and eliminate foreign elements. Characterizing antigen-antibody epitopes is fundamental to understanding pathogen-host interactions, but traditional approaches rely on protein purification techniques that are tedious and time-consuming. Cell-free protein expression systems present an exciting alternative to achieve parallel, high-throughput bio-safe production of antigens.
We used an innovative silicon-based microcompartments array to parallelize antigen expression and antibody epitope screening. Each compartment is genetically programmed by high-density surface-bound linear DNA templates as DNA-brushes coding for a given antigen. Following on-chip expression by a cell-free *E. coli* lysate, nascent antigens are efficiently captured on surface traps to facilitate sensitive detection. In a matter of hours and without the need for purification, the chip displays a panel of antigens that can be assessed for antibody binding.

With this approach, we studied the binding epitope of monoclonal antibodies, including a well-characterized antibody with a known epitope that was validated in our assay, and several antibodies whose epitope we characterize. We can assess relative affinity of antibodies to mutant antigens as well as absolute affinity by performing an antibody titration.
This approach allows to parallelly assess hundreds of antibody-binding targets, and its applications go beyond pathogen antigen recognition.

*Speaker

Modelling The Enzymatically Driven Rolling Motion Of Synthetic Cells

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Rolling motion has been shown to be relevant for the movement of viruses over cell surfaces. The exceptional speed, endurance, and directionality observed in self-rolling nanomotors demonstrate the potential of this movement strategy for bioengineering applications. Building upon this concept, RNase H-driven rolling of DNA-coated spherical particles achieves speeds and processivity far exceeding traditional DNA walkers. In our work, it was shown that this system can be transferred to induce the rolling motion of giant unilamellar vesicles (GUVs), a system that mimics the diffusivity and deformability of cell membranes. The study focuses on the modeling of RNase-catalyzed motion of GUVs and investigates the influence of different factors such as DNA linker lengths, RNase concentrations, and lipid compositions on GUV motion. It was observed that variations in these parameters significantly affect the motion of the GUVs, with the analysis of the mean squared displacement (MSD) showing a potentially superdiffusive behavior indicative of active processes. Simulations of one- and two-dimensional diffusive motions were performed to investigate possible analysis methods for the experimental data and a tracking algorithm was implemented to capture precise GUV trajectories and allow their analysis. The research lays the foundation for understanding the complex dynamics of enzymatically driven motion of synthetic cells and proposes a detailed analysis for a comprehensive understanding of these systems, which is of additional relevance for virology.

*Speaker

Immune nanoghosts for advanced cancer therapy

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Treatment of cancer, with cellular sensitivity, needs to be coupled with new therapeutic modalities that can target and treat single cancer cells throughout the body. Recent success of immunotherapies in cancer treatment has highlighted the promise of this approach, however, in some patients the natural population of immune cell is either depleted or incapable of detecting and killing the target cancer cells. Moreover, personalized cell-based immunotherapy approaches are very expensive and time consuming. In this multidisciplinary research we aim to develop modular bioinspired synthetic immune vesicles that target and treat cancer cells inside the body. Our recent studies on bottom-up assembly of synthetic cells have contributed novel engineering strategies to reverse engineer and understand cellular immune phenomena as molecularly defined systems. However, the implementation of fully synthetic immune systems for in vivo applications is limited due their clearance by the body immune system. To overcome this limitation, in our study we combine top-down and bottom-up synthetic cell approaches to produce immune red blood cell-based nanovesicles. We show how the surface of these nanocarriers can be modified with different cytotoxic proteins, yet their essential biomimetic properties still to be present. We provide initial in vitro data presenting the efficiency of these nanocarriers against cancer cells and their interaction with macrophages.

*Speaker

Microfluidic technologies for artificial cell membrane engineering

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Engineering biological devices that can emulate capabilities of living cells from molecular building blocks is a longstanding goal of synthetic biology. In recent years great progress has been made in recapitulating sophisticated cytosolic processes from purified components, such as the ability to regulate protein synthesis. In contrast, artificial cell membranes lag behind in their capabilities due to a poor understanding of how to engineer membrane complexity, for example, by introducing functional membrane proteins. Among the many outstanding challenges, one is the lack of precision techniques to systematically explore the multidimensional spaces of membrane composition parameters like lipid content. In consequence, relationships between critical membrane processes, e.g. protein binding and inserting, and lipid composition are poorly characterized. To alleviate this need, we present the development of suite of microfluidic tools to build, manipulate artificial cells with different membrane composition and quantify their biophysical properties. Advances in our microfluidic tool-kit includes devices for on-the-fly variation of artificial cell lipid composition and multiplexed perfusion over immobilized artificial cell populations. Furthermore, we add to our compositional probes by developing a DNA based optical sensor for assaying membrane surface charge.

*Speaker

Cell-free expression localized and activated at heated air-water interfaces

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The reaction networks that define modern cells and maintain their living functions have been finely optimized throughout their evolutionary history. Their components are kept at the working concentrations either by metabolic synthesis or uptake from the surrounding environment through a complex transmembrane transport machinery. But how could such a wide array of molecules come together in the first place to kick start the first cell-like reactions? Moreover, how could the concentrations required for them to interact be maintained without active transport and production by the cell itself? We propose a simple physical mechanism that is able to accumulate all the molecular pieces required for RNA transcription and protein translation, two fundamental cellular reactions, from a disperse and inactive mixture and locally confine them for long periods of time. By applying a temperature gradient to an air-water interface a local water cycle is created. Molecules in the solution are concentrated at the warm side of this interface by the combined effects of microscale evaporation and capillary flows. The findings not only shed light on a possible mechanism by which the first archaic reaction networks could have formed but it will also allow to create interacting and cooperating multicellular expression systems without the barrier of cell membranes and offer a novel paradigm for long-term feeding of transcription-translation systems.

*Speaker

Artificial Organelles Encapsulating Autocatalytic Enzyme Reactions For Application In Controlled Release

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Nanoparticle-based drug delivery systems (nanomedicines) provide a promising method to tackle many of the problems associated with conventional drug delivery. Most of the advantages of nanomedicines can be attributed to enhanced temporal and spatial control of the active pharmaceutical ingredient (API) within the body, allowing controlled and targeted drug delivery. Several stimuli responsive systems have been created, reacting to triggers such as light, pH and temperature, however approaches that respond to metabolites in their local environment are under considered.

This work demonstrates a novel mechanism for controlled release, utilizing liposomes, the most clinically well-established nanoparticles, to encapsulate urease and create a synthetic organelle that can sense and respond to metabolites. Urease liposomes respond to external urea, triggering a responsive mechanism of temporally controlled drug release. Control is then derived from the ability to manipulate the urea-urease reaction's clock-like behaviour, where changes from low to high pH can be used as the intrinsic stimuli to trigger drug release. By changing properties, such as enzyme concentration, lumen pH and membrane composition, the environmental response and drug release profiles of these artificial organelles can be manipulated.

We use the oncology drug Doxorubicin as a model weakly basic drug that can be actively loaded into liposomes down a pH gradient. During loading, Doxorubicin molecules readily diffuse across the liposomal membrane, where they reach the acidic pH of urease loaded liposomes, becoming protonated and 'trapped' within the liposome. The pH switch from the encapsulated urease reaction can then be used to reverse this process, increasing the pH in the lumen, deprotonating doxorubicin and allowing liberation from the nanoparticle. This work demonstrates a system where urease-doxorubicin nanoparticles show tuneable release in the presence of urea concentrations found under physiological conditions, achieving both delayed-release and constant-release profiles by varying formulations.

*Speaker

Protein Design For and With Synthetic Cells

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Proteins are the most versatile but challenging functional modules of Synthetic Cells (SynCells). When taken out of their cellular context, they often do not function at all or show unexpected behaviors, complicating the controlled engineering of SynCells for specific applications. Latest advancements in Protein Design promise to overcome this fundamental limitation: instead of repurposing proteins that were evolutionarily optimized in and for certain organisms, researchers can now attempt to design functional proteins specifically tailored for their *in vitro* system. This allows to progress towards more controlled and hypothesis-driven experiments, promoting SynCells as microscale machines that can be deterministically engineered.
In reverse, SynCells provide Protein Designers with the experimental means to translate recent computational advancements to the bench. As new computational methods allow the design of proteins with complex functionalities, experimental screening pipelines for more involved functions are urgently needed. SynCells provide exactly this: well-controlled environments to investigate complex behaviors of proteins, and established protocols to screen for a broad range of functions. The logical next step is now to utilize them to screen designed proteins.
Here, we present proof-of-principle of Protein Design both for and with SynCells. On one hand, we introduce a computational and experimental pipeline to generate self-assembling proteins that may serve as a cytoskeleton in SynCells, allowing to finely control mechanical properties to investigate biophysical theories of filament-based membrane deformation. On the other hand, we introduce a SynCell-based screening pipeline to test for i*n vivo* function of designed proteins, which finds computationally generated variants that can fully replace the wildtype. Further, we present how this pipeline can be utilized for a variety of functions. Taken together, we present the foundation to synergistically combine Protein Design and SynCells, bringing synthetic biology to the next level where complex biological systems can be computationally designed and experimentally screened.

*Speaker

Exploring the Role of Phospholipid Biosynthesis in Synthetic Cell Development: Insights into PlsB-PlsC Interactions

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Self-reproduction is a key feature of any living self-sustaining organism, and in this process membrane expansion and division are essential aspects. Phospholipids, the membrane building blocks present in all domains of life, play a central role. They form a barrier surrounding a confined compartment, creating a dynamic environment, in which specific phospholipids facilitate the functionality of proteins involved in metabolic processes (e.g. metabolite uptake and export, energy regeneration, substrate conversion). Therefore, any approach towards the bottom-up construction of a synthetic cell should involve a phospholipid membrane. In bacteria, especially in Escherichia coli, phospholipid synthesis starts with the acyltransferases PlsB and PlsC, responsible for the formation of the phospholipid precursors lyso-phosphatidic acid (LPA) and phosphatidic acid (PA), respectively. The involved enzymes have been studied individually, but not yet as a coupled reaction cascade while there is evidence for potential complex formation of these proteins, indicating a possible tandem biosynthesis of PA. Our goal is to further elucidate on the nature of the PlsB-PlsC interaction during phospholipid biosynthesis, through structural elucidation of these proteins in a native-like environment. Additionally, we are interested in regulatory mechanisms, in which we particular focus on the interplay between membrane expansion and cell division in the context of the synthetic cell.

*Speaker

Building environmentally interactive membrane systems using light responsive DNA nanostructures

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Membrane bilayer structures are vital for cell compartmentalisation within biological systems. DNA nanostructures can be modified to incorporate into membrane bilayers to direct, shape, and span lipid membranes. Modified DNA-liposomes have the potential to build interconnected synthetic ex vivo systems that can demonstrate dynamic membrane aggregation, communication, and morphological manipulation through DNA mediated interactions like strand displacement and switching. Dynamic DNA-liposome systems provide new methods for targeted delivery of molecular payloads through directed membrane aggregation and fusion. We designed a double stranded DNA nanostructure that is modified with cholesterol to allow for anchoring to liposome membranes. This nanostructure is further modified with photocleavable elements in the sugar-phosphate backbone that can break in the presence of UV light, creating fragments in one strand that dissociate to expose single stranded binding sites on the nanostructure. Displacement interactions were observed via SDS-PAGE gels, and confirmed by fluorescence microscopy. Using modified DNA nanostructures with light-interacting moieties like photocleavable elements, we can spatiotemporally regulate membrane surface binding between membranes in response to environmental changes, specifically the presence of UV light inputs.

*Speaker

Interdependent Metabolic and Genetic Networks show emergent properties in vitro

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A hallmark of all living organisms is their ability for self-regeneration which requires a tight integration of metabolic and genetic networks. Here we constructed a metabolic and genetic linked *in vitro* network (MGLN) that shows life-like behavior outside of a cellular context and generates its own building blocks from non-living matter. To this end, we integrated the metabolism of the crotonyl-CoA/ethyl-malonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle with cell-free protein synthesis using recombinant elements (PURE). We demonstrate that the MGLN produces the essential amino acid glycine from inorganic carbon (CO2), and incorporates it into target proteins following DNA-encoded instructions. By programming genetically encoded response into metabolic networks our work opens new avenues for building advanced biomimetic systems that show self-regeneration, decision-making, as well as evolution.

*Speaker

Building a living synthetic cell from non-living parts and how we plan build an even more synthetic one

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In 2010 scientists at the J. Craig Venter Institute (JCVI) constructed a living bacterial cell with a chemically synthesized genome. To do this it was necessary to develop new synthetic biology technologies including: (1) rapid methods for efficient whole genome synthesis from synthetic oligonucleotides, (2) a method to clone whole bacterial genomes as yeast centromeric plasmids and, (3) genome transplantation where a synthetic genome is installed in a suitable recipient cell to create a new cell with the genotype and phenotype of the synthetic genome. The cell we built, called JCVI-syn1.0, was widely called the first synthetic cell. By current definitions of what constitutes a synthetic cell, JCVI-syn1.0 is not one because in the genome transplantation process the synthetic genome is installed in a living bacterial cell. I will explain how we have modified genome transplantation so that a synthetic genome is installed in a recipient cell we have killed using the anti-cancer drug Mitomycin C, which destroys the genome of the recipient cell. Additionally, I will talk about how we are now working on building a living synthetic cell by combining a synthetic genome and a bacterial cell-free transcription-translation system in a synthetic lipid vesicle. I will also discuss why we think this approach may yield a living cell and why we fear it may not.

*Speaker

On the way to creating conductive GUVs

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One of the remarkable features of *Shewanella oneidensis* is that it can sustain anaerobic metabolism by giving away electrons to solid minerals instead of oxygen. In other words, these bacteria "breathe metals" in a process termed extracellular electron transfer (EET). The facultative anaerobes even connect to one another by special conductive appendages called nanowires, which enables them to upscale EET to tens of microns. Key machinery for this process are outer membrane proteins, which are located on membranes and membrane protrusions. We focus on the MtrCAB complex in particular and engage it in a minimal setup to better understand the mechanism of nanowire formation and mimic EET. To this end, we purify proteins, label, and reconstitute them in giant unilamellar vesicles (GUVs) by reverse-phase evaporation and electroformation. Fine tuning of experimental protocols results in homogeneous protein distribution and under certain conditions to nanowire-like structures, whereby efforts to deconvolute the influence of MtrCAB on the latter are made.

*Speaker

Bottom-up manufacturing of stabilised shaped lipid vesicles as scaffold for therapeutic artificial cell systems

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Red blood cells (RBCs) are our body's ultimate long-circulating delivery vehicles. Therefore, their biomimicry is increasingly receiving attention in biopharmaceutical research to develop advanced drug delivery systems. To disguise an advanced drug delivery system as an RBC, the composition, morphology and mechanics need to be mimicked. For the former, RBC mimics based on giant unilamellar lipid vesicles (GUVs) could prove ideal, but such structures lack the RBCs' biconcave shape and mechanical properties. To develop an enhanced RBC-mimicking GUV, we are expanding and developing novel microfluidic techniques to produce GUVs with a discoid shape, as it has been shown that discoid microparticles also display superior circulation behaviour. Next, we will be internally stabilising this shaped GUV with a cortex-like structure conferring the GUVs with RBC-like properties. We will present the first steps that have been taken for the reconstitution of a hydrogel and spectrin-based cytoskeletal cortex in shaped GUVs.

*Speaker

Cell-free Compartmentalized Synthesis of Membrane-associated Protein, PspA, Induces Shape Deformation and Pore Formation in Cell-sized Liposomes

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Phage-shock protein A (PspA) is a membrane-associated protein that is believed to play a critical role in bacterial membrane fusion, yet, its mechanism is less understood. In this study, instead of using purified PspA, we established cell-free PspA synthesis within liposomes and observed its phenotypic effects on membranes. This process is highly critical for the development of a self-sustained artificial cell model with cell-like properties induced by self-synthesized proteins that are generated from its genetic level. In this study, we successfully designed multiple plasmids of *pspA* and translated them to PspA using cell-free protein synthesis (containing *in-vitro* transcription and translation molecules extracted from *E. coli*) in both bulk and liposomes. In particular, PspA contains 5 α-helices (α1-α5); and here, the process of synthesis of each truncated α-helix was also successfully demonstrated. Moreover, cell-free synthesis of PspA (full-length, α1, α1-α2, and α1-α3) in a bulk system revealed aggregation and oligomerization (self-assembly) and formed μm sized filament-like structures. Nevertheless, such structures were not observed when synthesized within liposomes. Interestingly, though, it induced the shape change in liposomal membrane to be more elongated. This result implies that lipids may somehow interact and dissociate the PspA aggregates. By fluorescent leakage essay using GFP and calcein, we also observed that such PspA proteins (full-length, α1, α1-α2, and α1-α3) could induce leakage on membranes, indicating it might form small-sized pores on membranes. Overall, we highlight that α1 plays vital roles in PspA aggregation/polymerization, shape deformation, and membrane leakage. We assume that both shape deformation and membrane leakage may be the intermediate process before membrane fusion. The mechanism by which PspA or α1 form pores and membrane deformation is currently under further investigation.

*Speaker

Fungal hydrophobins as building blocks for pure protein bilayers and vesicles

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The compartmentalization of aqueous solutions is a fundamental requirement for living organisms, which is primarily achieved by the formation of a phospholipid bilayer membrane. However, the mechanical and biochemical properties of phospholipids are limited, necessitating alternative building blocks for use in applications such as biomedicine and synthetic biology. Proteins, due to their biocompatibility on the one hand and versatility through genetic engineering on the other, are promising candidates. In particular, hydrophobins, a family of strongly amphiphilic proteins, appear to be well-suited for these purposes. This study examines the assembly process and mechanical properties of films formed by fungal hydrophobins. These proteins self-assemble at water-interfaces to create stable monolayer films with a hydrophilic and a hydrophobic side. By contacting two interfacial films with their hydrophobic sides, stable bilayer membranes resembling lipid bilayers can be produced. These hydrophobin bilayers exhibit a similar thickness to lipid bilayers but can withstand much higher lateral tension (1) and are virtually impermeable to water (2). Furthermore, the hydrophobin bilayers can incorporate ion channels such as gramicidin A, which have been shown to be fully functional (3). The bilayers can also be formed between oily compartments by contacting the hydrophilic sides, enabling the formation of vesicles from both types of bilayers (aqueous and oily phases) using techniques such as microfluidic jetting. These pure protein vesicles may serve as an alternative platform for synthetic cells and provide a different matrix for the incorporation and study of membrane proteins.

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(2) Nolle, F. et al., Langmuir 39, 13790 (2023).
(3) Hahl, H. et al., Adv Mater 29, 1602888 (2017).

*Speaker

Supramolecular peptide designs for biomimicry: non-canonical membranes and functional cytoskeleton mimics

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The design of synthetic peptides with self-assembling properties is opening access to a plethora of new supramolecular structures and functions not evolved by nature. Our understanding of the basic rules that translate peptide sequence into defined assemblies have allowed chemists to rationally design minimalistic peptide scaffolds with biomimetic structure and function.
Here, we report two types of synthetic peptides able to imitate the role of certain cellular components through supramolecular self-assembly in water: (1) **Cyclic peptides** could organise hierarchically by sequential one-to-two dimensional (1D-to-2D) self-assembly, initially generating 1D nanotubes that further associate as 2D nanosheets. This novel 2D architecture, consisting of aligned hollow nanotubes, represents a non-canonical mimic of the cellular membrane with valuable self-healing properties, providing a new structural role to peptides not found in nature. (2) **Linear peptide amphiphiles** were confined in droplets and induced to self-assemble as fibrillar networks that imitate the structure of a natural cytoskeleton. This cytoskeleton mimic could trigger biomimetic responses, such as selective molecular uptake, membrane fusion and chemical communication between droplet populations. Overall, we show how rational peptide design for self-assembly can unlock new biomimetic structures and cell-like behaviour.
References: (1) J. Am. Chem. Soc. 2020, 142, 300–307. (2) Nat. Commun. 2021, 12, 6421.

*Speaker

Artificial spores as multi-functional biocatalysts to perform chemical cascades

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With the aim of creating a synergy between whole-cell biocatalysis and enzyme immobilization, artificial spores emerge as enhanced and tuneable biocatalysts. Inspired in nature, we hypothesized that artificial spores could surface as a tool to overcome a wide range of hindered biocatalytic pathways, in addition to grating stability to the systems. In this work, we created artificial spores of Escherichia coli heterologously expressing an alcohol dehydrogenase (ADH), coated with a tannic acid and iron chloride complex (TA-Fe(1)) where the immobilization of a transaminase (TA) was performed through different approaches. To validate the formation of the spores, we performed confocal laser scanning microscopy (CLSM) using fluorescence protein labelling (GFP, mCherry) as model proteins to confirm that proteins can be compartmentalized inside and outside of artificial spores. Furthermore, to validate the formation of the artificial spores, we performed several characterization techniques such as XPS, ICP-MS, SEM, TEM, and AFM, further validating the manufacturing of these spores. Finally, we tested the biocatalysts in a multi-enzymatic model cascade, 4-hydroxybenzylalcohol to 4-hydroxybenzylamine, where the successful amination of an aromatic alcohol was achieved. Therefore, this study demonstrates the ability to manufacture artificial spores and their viability for biosynthetic purposes.
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*Speaker

Nucleus: an open-source, full-stack distribution that lets you start developing with synthetic cell systems today

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Fully defined, encapsulated synthetic cells are a highly engineerable, cell-like complement to traditional cell free systems. Engineered synthetic cellular systems can enable unconstrained development of non-natural biomolecules, highly controllable environments for candidate molecule prototyping, and safely deployable evolution-resistant biology. More broadly, synthetic cells have the potential to replicate and surpass the capability of natural cells. To achieve this goal, working with synthetic cells must be routine, reliable, and accessible. Unfortunately, this is not now the case: synthetic cell engineering remains a bespoke, high-complexity endeavor. Getting started can take years and require direct correspondence with the academic labs originating specific technologies. Key materials can be difficult to source: for example, the available gene sets required to produce the PURE system are incomplete or only available under restrictive licenses. In collaboration with Schmidt Futures & Caltech, we are developing an open-source, full-stack distribution for rapidly booting up and routinely engineering synthetic cells. We are refactoring, optimizing, and integrating now disjoint and incomplete protocols and materials and building complementary design and analysis tools to make synthetic cell engineering reliable, reproducible, and accessible. Our open-source distribution, Nucleus v0.1.0, enables research groups to quickly begin engineering with synthetic cells using integrated and validated protocols, materials, and tools for (1) cytosol production, (2) DNA module implementation, and (3) liposome encapsulation. We are developing and releasing increasingly capable synthetic cell tools and materials to support researchers and engineers and are excited to work with others to routinize synthetic cell engineering

*Speaker

An artificial respiratory chain powering synthetic CO2 fixation

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Photosynthesis, which fixes CO2 on a global scale, is naturally organised in the chloroplast. This organelle hosts the highly integrated nanostructures which convert light into chemical energy that in turn powers CO2-fixation. The working principles of photosynthesis have recently been recapitulated *in vitro* in a synthetic 17-enzyme CO2-fixing cycle named CETCH, which provides a new-to-nature solution that improves upon natural CO2 fixation. In the past, natural thylakoid membranes isolated from spinach have been used to power this cycle; however, to build a truly artificial platform, energy modules that are directly tailored to the cycle demands are needed. Here we couple the CETCH cycle to proteoliposomes (biochemically defined, synthetic membrane vesicles reconstituted with purified proteins), establishing an energy conserving respiratory chain that can synthesise ATP and power CO2 fixation. We discuss how proteoliposome platforms can increase the complexity of *in vitro* synthetic metabolic networks and support the construction of complex, self-powering nanostructures and synthetic organelles that offer alternatives to naturally evolved systems.

*Speaker

Deciphering genome transplantation mechanisms as a step towards understanding basic principles of life

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Building a cell from the ground up would help identify the minimal set of elements necessary for a cell-like compartment to become a functional living entity. This requires an extensive understanding of the contribution of each component to the cell function. This project focuses on genetic information and its processing by a compartment capable of gene expression, using an original approach named whole genome transplantation (WGT). This technique consists in isolating a whole bacterial genome belonging to Species A (donor genome) and installing it in the cytoplasm of Species B (recipient cell), resulting in cells genotypically and phenotypically identical to Species A.
WGT is currently performed on *Mollicutes*, the simplest living forms capable of autonomous replication outside of a host, an ideal model for studying essential requirements for life. Understanding what defines the compatibility between the donor genome and recipient cell may lead to identifying key elements that enable booting up a living cell and to understanding the rules that regulate the interactions between genetic material and the compartment which expresses it. We hypothetize that the ability of the recipient transcription machinery to "interpret" the data encoded on the donor chromosome is essential for successful boot-up. Our approach consists in engineering a recipient cell, *Mycoplasma capricolum (Mcap)*, to preload it with transcription factors belonging to a donor genome, *Mesoplasma florum (Mflorum)*.
The coding sequences of the five subunits of the *Mflorum* RNA polymerase were cloned into plasmids along with their native promoters. Transformation of *Mcap* suggests that the *Mflorum* genes can be expressed individually in *Mcap* and that their presence does not impact the recipient's survival. In the next phase, the 5 subunits will be simultaneously expressed in *Mcap*. The resulting cells will be used as recipients for WGT assays and structural analysis of their transcription machinery will be performed

*Speaker

Isolation of tRNA pools for cell-free translation systems

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Transfer RNAs act as the key substrates for translation by decoding the genetic code into protein. As such, the tRNA pool is a critical reagent in cell-free translation systems (CFTS) such as PURE and lysate-based TxTl expression systems. Usually, tRNA pools for CFTSs are sourced commercially from a scant number of vendors which represents a key vulnerability should supply-chain issues arise. Further, commercially available tRNAs for CFTSs are limited to the *E. coli* strain MRE600 which may be inappropriate for the translation of eukaryotic proteins or use in cell-free translation systems of non-model organisms. To address these challenges, we have employed previously described methods which generate tRNA pools from various host strains. We find that these methods provide an inexpensive and convenient source of tRNA pools for CFTSs that perform as well or better than commercial sources. We expect these techniques will enable further research in cell-free translation systems ranging from PURE to non-model, lysate-based systems.

*Speaker

RNA aptamers for lipid membrane anchoring of RNA origami nanopores

Tim Karrasch * [1,2,3], Erik Poppleton [1,3,4], Alessandra Griffo [1,3], Saúl Sandoval Hernandez [2], Ebbe Sloth Andersen [5], Frauke Graeter [4], Kerstin Goepfrich [1,3]

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Nucleic acid-based nanotechnology plays an increasingly important role in the field of synthetic biology, as it enables the bottom-up design of synthetic cell-like components. Many of these components, most prominently pores, require an insertion or association to lipid membranes. Previously, DNA-based membrane active components were developed and inserted into membranes using chemical functionalization with cholesterol tags. Instead, we would like to expand the synthetic biology toolkit by a purely nucleic acid-based lipid-binding aptamer, avoiding the need for costly synthesis, and allowing both in vitro and in vivo production of our custom-made molecular hardware.

Here, we present a specific RNA sequence that can bind and permeabilize liquid-phase phosphatidylcholine membranes, based upon a previously identified set of sequences.Membrane permeability for otherwise impermeable substances induced by the RNA structureis verified and characterizedby ionic current recordings,dye influx and dye releaseexperiments. The mechanism of this interaction is investigated by rational modifications of the structure, yielding more efficient and truncated variants.

We aim to combine the shown aptamer with an RNA origami pore. So far, we have already demonstrated successful folding of the pore, verified by cryo-electron microscopy and molecular dynamic simulations. Moreover, insertion of the transmembrane pore and validation of its function was already achieved by traditional cholesterol functionalization.

The demonstrated results could have an impact beyond its application in nucleic acid-based nanotechnology as it could help elucidate, in the context of the RNA World hypothesis, how membrane permeability could have been established in an RNA World.

*Speaker

Shape instability of synthetic cells by intracellular wetting

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Biomolecular condensates organize intracellular processes in space and time. The interfacial tension of the droplets generates capillary forces on objects upon contact, known as wetting phenomena. Despite the understanding of the compartmentalization properties of condensates, the biological functions of wetting remain largely unexplored. Here, we developed synthetic cells based on a bacterial cell-free expression system, that reconstitutes intracellular wetting. By inducing crowding via evaporation in the cell-sized spaces, the cell-free cytoplasm undergoes liquid-liquid phase separation. To study wetting on the cytoskeleton, we expressed the bacterial cytoskeleton MreB with simultaneous phase separation. Droplets nucleate and wet on the cytoskeleton, promoting the aggregation of the cytoskeleton via capillary interaction. Finally, by incorporating the deformable membrane into the system, we investigated the interplay of condensates, cytoskeleton, and membrane. We found that the intrinsic cortex formation of MreB on the membrane was followed by wetting, resulting in interfacial wrinkling of the cells. These results suggest that the wetting properties of droplets provide a novel means for the morphological control of synthetic cells.

*Speaker

Construction of artificial cells that self-produce lipids inside

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The construction of living cells from molecules and genes is a big challenge in life science and bioengineering. Because creating cellular life in the laboratory evokes the process of the birth of early life, building cells may lead to the understanding of universal rules that allow the emergence of life. Several essential cellular functions have been reconstituted from scratch so far, in vesicles or in vitro. However, building the lipid synthesis process inside the membrane is still challenging. This difficulty is one of the reasons why the reproduction of cell self-propagation is still not achieved in artificial cells. Here, we constructed phospholipid-synthesizing artificial cells by combining fatty acid synthesis system and a cell-free system. The fatty acid molecules were generated by 9 kinds of recombinant proteins and, subsequently, transformed into phosphatidic acids by acyltransferases which were synthesized by the cell-free gene expression. So designed system was assembled inside giant vesicles and produced 10 % of the phospholipids of the mother cell membrane. This reconstructed system would be a platform for the creation of self-reproducing artificial cells.

*Speaker

Exploration of tryptophane indispensability as a pathway towards a reduced amino acid code

Vítězslav Kučera * [1], Valerio Giacobelli [1], Tereza Neuwirthová [1], Klara Hlouchova [1]

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Cellular life as we know it is dependent on all 20 canonical amino acids comprising the majority of all proteins. Study of origins of life have implied, that life might have begun its existence with a smaller alphabet. Although imaginable as a hypothetical concept, providing experimental evidence for such organism has proven to be a difficult task, as our understanding of life's many intricacies as well as rational protein design is lacking. Recent advancements in the combinatorial genome editing and in silico protein folding serve as a stepping stone on the path towards experimental reductions of amino acid code. For a first step in this reduction, we chose tryptophane as a suitable candidate as this amino acid is currently thought to be a later addition to the alphabet, it is one of the least represented amino acids (comprising _˜1.5 % of AAs and having one codon) and rarely participates in the active site of enzymes. Our proof-of-concept experiment on the tryptophane operon genes of *Escherichia coli* using Multiplex Automated Genome Engineering (MAGE) showes, that not only is it possible to exchange all tryptophane sites with other amino acids without major disruption of phenotype, but we also gathered the data indicating suitable candidates for the massively parallel exchange of tryptophane in the whole genome. In silico predictions of favourable mutations also closely matched the observed phenotype indicating a possibility of rational design of such reduced organism.

*Speaker

From Context to Code: Rational De Novo DNA Design and Predicting Cross-Species DNA Functionality Using Deep Learning Transformer Models

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In the realm of synthetic biology, the traditional trial-and-error methodologies have long been a bottleneck, limiting the efficient engineering of organisms and the scalability of biomanufacturing processes. In this talk, I am going to present a novel DNA Design Platform, leveraging the predictive capabilities of Transformer-based deep learning models. This platform marks a departure from conventional practices, focusing on the context-sensitive and host-specific engineering of 5 regulatory elements-promoters and 5 untranslated regions-alongside a panel of codon-optimised coding sequence variants in a diverse array of expression hosts.

This approach represents a pivotal shift in the field, utilising advanced deep learning models to transform the traditional design, build, test, learn cycle into a more streamlined, predictive, and efficient framework. This approach significantly expands the range of achievable gene expression profiles and phenotypic outcomes, dramatically reducing the reliance on intensive high-throughput screening processes. The context-aware, AI-driven design strategy we present is not just an incremental improvement but a significant leap in synthetic biology. It offers a scalable, refined approach for gene expression optimisation across species barriers.

Overall, I will highlight how our research that showcases the significant capabilities of transformer models to increase both the accuracy and efficiency within synthetic biology. This shift from a predominantly trial-and-error methodology to a more data-centric strategy underscores the transformative progress in synthetic cell research. By adopting a data-driven approach, we are facilitating a transition towards more informed and precise decision-making in the creation and development of synthetic cells.

*Speaker

Thermoresponsive lipopolypeptide towards controlled aggregation on liposome surface

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Pore-forming proteins like α-haemolysine are dynamic systems made of inactive membrane bound proteins able to form multimeric transmembrane assemblies on demand, generating then transport across the cell membrane. Inspired by these proteins, a number of research studies are aimed at designing artificial systems, including polymers, that can create membrane permeability in the presence of ions or light, or following a change in temperature or redox conditions. Our work develops aminoacid-based polymer scaffold with inactive membrane bounding ability that are able to enhance and control membrane destabilization upon multimerization. This polymeric backbone is synthesized via the ring-opening polymerization of *N*-carboxyanhydrides monomers. This reaction affords the synthesis of a thermoresponsive lipo-polyproline by using phospholipids as initiator, a new class of polypeptides that were fully characterized by NMR, MALDI-TOF and turbidimetry analyses. To assess their membrane bounding ability, these biomimetic polymers were formulated into giant unilamelar vesicles through electroformation. By increasing temperature, a phase separation was observed by confocal microscopy at the surface of vesicles revealing polymer multimerization capacity. The permeability of the vesicles were also monitored by fluorescence, a result revealing that the polypeptides can be used to build synthetic cells with controlled passive transport across their membranes.

*Speaker

Formation and Properties of Fatty acid and Fatty Acid/Phospholipid Blended Coacervate-supported Membranes

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This presentation will discuss that how the presence of phases formed via liquid-liquid phase separation (LLPS) impacts fatty acid and/phospholipid blended membrane morphology, fluidity, and permeability and how the interactions between lipid and LLPS are tuned by varying lipid and LLPS compositions. Compartmentalization is crucial to how protocellular systems formed on the early earth. Even in modern cells, there are both membranous and membraneless compartments. These compartments are beneficial to the intracellular organization and facilitate cellular bioreactions by providing suitable chemical environments. To construct compartments, we used coacervate droplets formed by LLPS of charged polymers, and have observed templated lipid membrane self-assembly around these droplets. Although coacervates possess many advantageous properties such as spontaneous formation and selective accumulation of molecules, these structures are unstable against coalescing and sensitive to salt and pH. Therefore, the formation of lipid membranes at the interface of coacervate droplets via simple gentle hydration results in a structure that takes advantage of both a membrane boundary and macromolecularly crowded interior "cytoplasm". Permselective molecular transport is crucial for lipid membranes and beneficial for protocellular compartments. However, previous studies have also shown that the addition of single-chain fatty acids, which are considered more prebiotically-plausible, to phospholipid membranes increases membrane permeability to solutes. Greater permeability has been observed for higher amounts of fatty acid in the membranes. Moreover, fatty acid or fatty acid blended vesicles have shown low magnesium stability, which is a disadvantage for RNA compartmentalization and the "RNA World" hypothesis since Mg2+ commonly participates in RNA folding and catalysis. However, in the presence of LLPS interior, we observed the structural integrity and magnesium stability of the membrane were profoundly improved. Taken together, the resulting structure could be a great candidate as a protocell and synthetic cell model.

*Speaker

Light-based juxtacrine signaling between synthetic cells

Allen Liu * [1], Hossein Moghimianavval , Kyle Loi , Sung-Won Hwang , Yashar Bashirzadeh

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Cell signaling through direct physical cell-cell contacts plays vital roles in biology during development, angiogenesis, and immune response. Intercellular communication mechanisms between synthetic cells constructed from the bottom up are majorly reliant on diffusible chemical signals, thus limiting the range of responses in receiver cells. Engineering contact-dependent signaling between synthetic cells promises to unlock more complicated signaling schemes with different types of responses. In this work, we designed and demonstrated a light-activated contact-dependent communication tool for synthetic cells. We utilized a split bioluminescent protein to limit signal generation exclusively to contact interfaces of synthetic cells, driving the recruitment of a photoswitchable protein in receiver cells, akin to juxtacrine signaling in living cells. Our modular design not only demonstrates contact-dependent communication between synthetic cells but also provides a platform for engineering orthogonal contact-dependent signaling mechanisms.

*Speaker

Extended Lifespan for Synthetic Circuits in Polymersomes via Microfluidic Technologies

Hongfei Liu * [1], Jérémie Marlhens [1], Heinz Köppl [1]

[1] Technical University of Darmstadt – Germany

This study introduces a microfluidics-based approach for producing polymersomes encapsulating E.coli lysate-based cell-free systems for long-lasting circuit functionality. Our objective is to construct a genetic oscillator capable of continuous cell-free energy buffer replenishment through hemolysin channels within the polymer membrane, representing a significant step forward in artificial cells.
We utilize a diblock copolymer to make polymersomes that can undertake osmotic changes and support the insertion of membrane proteins. Although microfluidic methods offer many benefits and polydimethylsiloxane (PDMS) chips are easy to fabricate, several challenges including evaporation, material incompatibility, and incomplete dewetting hinder sustainable cell-free gene expression in polymersomes. By optimizing microfluidic devices and solvents, we facilitate prolonged observation of the internal dynamics and the responses on membranes of polymersomes.

Currently, dilution is the key method of enabling two crucial features for cell-free oscillators: it reduces transcription factor concentrations and replenishes energy. We aim to implement these functions with targeted protein degradation and alpha-hemolysin respectively, thus circumventing dilution. As the first step towards an encapsulated genetic oscillator, we successfully tested an oscillator in a batch cell-free system without degradation, exhibiting switch-like behavior and tunability with plasmid concentration and inducers. First, adding degradation is a crucial step to achieve oscillation. Second, integrating alpha-hemolysin channels into lipid membranes facilitates component replenishment through diffusion. We aim to transfer this ability to polymersomes, thus extending the longevity of the encapsulated cell-free system. Our novel method, i.e., using protein degradation to mimic dilution effects, has been validated by simulations.

This work takes crucial steps toward demonstrating the feasibility of genetic oscillators in polymersomes, a feat previously limited to living cells. Together with the high throughput and uniformity of polymersomes, this platform can be ideal for screening genetic circuits.
Keywords: Microfluidics, Polymersomes, Cell-free systems

*Speaker

Spatio-temporal control of nucleic acid catalysis in an active droplet system

Corbin Machatzke * [1], Anna-Lena Holtmannspötter *

[2], Hannes Mutschler [1], Job Boekhoven [2], Elia Salibi [1], Carsten Donau [2], Christian Begemann [1]

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Cells use transient membrane-less organelles to regulate biological reaction networks - for example, in response to heat or oxidative stress, stress granules selectively store mRNA to downregulate certain protein expressions. Models mimicking this active behavior should be established to better understand in vivo regulation involving compartmentalization. Here we use active, complex coacervate droplets as a model for membraneless organelles to spatiotemporally control the catalytic activity of a DNAzyme. Upon partitioning into our peptide-RNA droplets, the DNAzyme unfolds and loses its ability to catalyze the cleavage of a nucleic acid strand. Upon inducing droplet formation with fuel, we can transiently pause the DNAzyme activity. After fuel consumption, the DNAzyme activity autonomously restarts. We envision this system could be used to up and downregulate multiple reactions in a network, helping understand the complexity of a cell's biological pathways. By creating a network in which the droplet properties could be reciprocally regulated by the DNAzyme, we would have a powerful tool for the engineering of synthetic cells.

*Speaker

Sculpting DNA-based synthetic cells through phase separation and phase-targeted activity

Layla Malouf * [1,2], Diana Tanase [1,2], Giacomo Fabrini [2], Ryan Brady [3], Miguel Paez-Perez [2], Adrian Leathers [4], Michael Booth [5], Lorenzo Di Michele [1,2,6]

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The creation of structures comprised of chemically and physically distinct compartments is key to the bottom-up approach for constructing synthetic cells. Simple DNA nanostars, made amphiphilic through the addition of cholesterol moieties, have been shown to robustly self-assemble into networks with programmable structure and stimuli responsiveness. In this work, we show that mixed populations of these amphiphilic DNA nanostars, dubbed "C-stars", can self-assemble, undergoing phase separation to form condensates with multiple compartments. Molecular programming allows modification of these compartments to host phase-specific functionality and responsiveness. We use these binary condensates, along with other modular building blocks, to construct synthetic cells featuring a membrane, a cytoplasm, and organelles. Like eukaryotic cells, our synthetic cells can localise distinct processes in different sub-compartments and support life-like functions, including RNA synthesis and a disassembly response analogous to cell death. Our strategy could be exploited for the rational design of cell mimics with distinct and chemically addressable microenvironments, with applications including biosensing and drug delivery.

*Speaker

Interaction of In Vitro Transcribed RNA with Lipid Membranes

Adam Mamot * [1], Nikolas Wetzel [1], Jan-Hagen Krohn [1], Petra Schwille [1]

[1] Max-Planck-Institut für Biochemie = Max Planck Institute of Biochemistry – Germany

Biomimicry of minimal systems requires a sophisticated balance of their biomolecular elements, such as lipids, proteins, and nucleic acids. RNA is an excellent building block due to its structural and functional flexibility: RNA can dynamically change conformation (riboswitches), store genetic information (mRNAs), specifically bind ligands (aptamers), catalyze reactions (ribozymes), and form macromolecular scaffolds (RNA origami). Therefore, the rational incorporation of RNA into minimal systems seems inevitable in the context of bottom-up synthetic biology. On the other hand, RNA is structurally complex and inherently fragile, which makes its handling and application as a building block particularly challenging. Furthermore, the extremely complex interplay between RNA and other biomolecules is critical to its function and activity. Among these, the interactions between RNA and lipid membranes are still very poorly understood. Moreover, current studies are limited to interactions of lipid membranes with RNA oligonucleotides (obtained by solid-phase synthesis), which dramatically limits the scope of RNA properties and functions that can be investigated in this context. Here we present a systematic study of the intrinsic interactions of macromolecular RNA with lipid membranes. We used in vitro transcription (IVT) and bioorganic chemistry for the preparation of RNA molecules. This gave us access to RNA of unlimited length and a wide range of chemical modifications (e.g. fluorescent labeling). Lipid membrane models, such as small unilamellar vesicles (SUVs) and supported lipid bilayers (SLBs), were used to provide a flexible platform for the investigation of membrane properties (e.g. lipid phase, surface charge). Methods such as EMSA, DLS and fluorescence microscopy were used to gain a comprehensive insight into RNA-membrane interactions. Our initial data show promise for a deeper understanding of RNA behavior in the environment of the lipid membrane surface, providing a step closer to rational design of minimal systems involving compartmentalization and spatio-temporal control of RNA molecules.

*Speaker

Engineering Life-like Materials via Protocell Design and Dynamics

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Recent progress in the chemical construction of micro-compartmentalized semipermeable colloidal objects comprising integrated biomimetic functions is paving the way towards rudimentary forms of artificial cell-like materials (protocells) for modelling complex biological systems, exploring the origin of life, and advancing future proto-living technologies.
In this talk, I will demonstrate simple forms of individuated and collective behaviour in synthetic protocells and protocell communities. I will discuss: (i) new approaches to complex synthetic cells (bacteriogenic protocells) by living material assembly ("side-on" assembly) (1); (ii) enzyme-powered sensing, motility and oscillation in DNA-based protocells (2,3); (iii) oligonucleotide-based signal processing in protocell networks (4,5); and (iv) programmed assembly of beating or extendable prototissues (6,7). These studies offer new pathways towards artificial life materials capable of autonomic behaviour and programmable agency.

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*Speaker

A Genetic Unit to Enable Membrane Protein Insertion and Translocation on Synthetic Membranes

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[3] Max Planck Institute for Terrestrial Microbiology – Germany

Membrane proteins are essential elements of life, equipping biomembranes with diverse functions like mechanical strength, selective transport, energy production and signal transduction. However, their hydrophobicity and the complex machineries needed for membrane insertion present technical challenges. Although the key functionalities encoded by membrane proteins are compelling for academic and technological applications, membrane proteins remain underutilized *in vitro*.
We are developing a simple tool to facilitate the directional insertion and functionality of membrane proteins produced *in vitro*. By combining cell-free protein synthesis with a genetic unit for the *Escherichia coli* SecYEG system, we can assemble a functional translocon to promote the translocation and insertion of co-expressed reporters on intact synthetic membranes. Our entirely DNA encoded system obviates any protein overproduction, purification or reconstitution steps.

The genetic unit is both compatible with lysate based expression systems and PURE and can be customized with auxiliary gene modules, like the motor ATPase SecA and the membrane insertase YidC. Our system improves the insertion of integral membrane proteins and furthermore enhances the orientational bias of insertion compared to spontaneous insertion.
Combined with a real-time translocation assay based on split NanoLuc, the system allows for high-throughput screening of several hundred SecYEG variants in parallel. Since the entire system is implemented *in vitro*, we can now investigate variants that are otherwise lethal or those specifically beneficial for applications outside of living cells. Our tool, delivered as DNA, is easy to implement and adapt for a wide variety of *in vitro* membrane applications.

*Speaker

Seven governing principles in biology

Tae Seok Moon * [1]

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In physical science such as physics and chemistry, there are governing principles that are universal and applicable to all relevant systems, including energy conservation, entropy increase, uncertainty principle in quantum mechanics, and chemical equilibrium. However, what are governing principles in biology that are unique to all living systems? After collecting opinions and thoughts from diverse scientists and engineers all over the world, I summarize seven governing principles or laws in biology: Central dogma, evolution, biological robustness, regeneration, reproduction, development, and causality. Some of these are not necessarily unique in biological systems from a reductionist's point of view (e.g., causality), and others are applicable predominantly to eukaryotes (e.g., reproduction and development). Notably, many engineering systems have mimicked biological systems to enhance their performance. In this perspective article, I discuss these principles to better understand the rules of life and help construct improved engineering systems that we can use and control in an ethical, safe, and rational way.
Tae Seok Moon, *Front. Synth. Biol.*, 03 November 2023

Sec. Genetic Systems and Circuit Design
Volume 1 - 2023 — https://doi.org/10.3389/fsybi.2023.1296513

*Speaker

In vivo functionality and biocompatibility testing of non-invasively trackable stealth liposomes in Galleria mellonella

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With the aim of achieving long-circulating synthetic cells, such as synthetic red blood cells, devising an imaging approach for cell tracking early-on is essential for translation towards clinical studies. Moreover, as a heavy ethical and logistical burden is posed on the use of small animal models, there is a need for an intermediate model that could bridge initial *in vitro* and *in vivo* functionality and biocompatibility screenings. Therefore, we aim to (i) design biocompatible stealth liposomes with a non-invasive, cost-effective and feasible imaging strategy for the tracking of long-circulating lipid-based vesicles and (ii) establish *Galleria mellonella,* owing to its innate immune system relating to that of mammals, as a relevant intermediary model for initial biocompatibility and imaging functionality screenings. To achieve this, we have developed fluorescent (DiD dye), microCT contrast-enhanced (Iodixanol) and multimodal stealth liposomes and subsequently evaluated their biocompatibility and imaging functionality *in vivo* using larvae of *G. mellonella*. We thereby deliver a proof-of-concept for the initial functionality and biocompatibility screenings of trackable stealth liposomes in an intermediary model.

*Speaker

Mimicking plant biosynthesis potential through Synthetic Microbial Communities

Fabian Moreno-Avitia * [1], Erika Gonzalez *

, Juan Nogales *

[1] Centro Nacional de Biotecnología [Madrid] – Spain

The current targets of metabolic engineering are associated with lengthy, intricate, and energy-demanding pathways, akin to those found in plants. Among these targeted compounds, flavonoids hold a high significance. This pathway competes with fatty acid biosynthesis and depletes products from the aromatic amino acids pathway. Despite successful flavonoid production in microorganisms, these projects overlook one of the main advantages of plant biosynthesis: sustainability achieved through the use of CO2 as the sole carbon source. To achieve this complex objective, we employed the concept of distributed catalysis by constructing a synthetic microbial community. This community encompasses all the components of the flavonoid production pathway, segmented into farming, precursor, and assembly modules. The farming module comprises an engineered strain of *S. elongatus* responsible for CO2 fixation and sucrose production, serving as the secondary carbon source of the system. In the precursor module, p-coumaric acid is synthesized by a modified strain of *E. coli*. Additionally, this bacterium hydrolyzes sucrose, releasing fructose exclusively for consumption by the assembly module. Finally, the assembly module, consisting of engineered *P. putida*, assimilates fructose and the precursor to generate Naringenin, the flavonoid diversity "kernel". The primary objective of this community is to mimic plant flavonoid production by incorporating its most pertinent features to establish an innovative bioprocess. Furthermore, we have demonstrated the potential of distributed catalysis in enhancing the development of new sustainable biotechnological solutions applicable across a wide range of targets.

*Speaker

Leveraging active learning to establish efficient in vitro transcription and translation from bacterial chromosomal DNA

Leonardo Morini * [1], Wilhelm Huck [1]

[1] Radboud university [Nijmegen] – Netherlands

Gene expression is a fundamental aspect in the construction of a minimal synthetic cell and the use of chromosomes will be crucial for the integration and regulation of complex modules. Expression from chromosomes in in vitro transcription and translation (IVTT) systems presents limitations, as their large size and low concentration make them far less suitable for standard IVTT reactions. Here, we addressed these challenges by optimizing lysate-based IVTT systems at low template concentrations. We then applied an active learning tool to adapt IVTT to chromosomes as template DNA. Further insights into the dynamic dataset lead us to adjust the previous protocol for chromosome isolation and revealed unforeseen trends pointing at limiting transcription kinetics in our system. The resulting IVTT conditions allowed a high template DNA efficiency for chromosomes. In conclusion, our system shows a proteins-to-chromosome ratio that moves closer to *in vivo* biology and represents an advancement towards chromosome-based synthetic cells.

*Speaker

Cyclic peptide self-assembly towards potential membrane mimics

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In the engineering of synthetic protocellular systems, peptides have emerged as versatile tools capable of replicating numerous cellular structures and functions, ranging from mimicking the dynamic behaviour of the cytoskeleton to orchestrating complex intracellular signalling pathways. A relatively unexplored application of peptides in protocell engineering is their assembly as cell membrane mimics, which constitutes a non-canonical role of peptides naturally performed by lipids as cell boundaries.
This study introduces an amphiphilic cyclic octapeptide (**CPx**) designed to self-assemble into one-dimensional (1D) nanotubes, which subsequently arrange as tubular bilayers in aqueous environments, generating supramolecular 2D nanosheets (Fig. 1). The hierarchical 1D-to-2D organisation of peptide nanosheets results in a structural mimic of lipid membranes, partitioning amphiphilic domains in an aqueous environment. Reversible transitions between assembled, dispersed and aggregated states of the nanosheets provide self-healing properties that can be controlled by external stimuli.
Overall, we here report a novel design for 2D peptide self-assembly, while also demonstrate a noncanonical structural role for peptides, not evolved by nature as membranes.

*Speaker

Europe's Synthetic Biology Innovation Ecosystems: Insights from the SYNBEE Project

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Background: The SYNBEE project investigates synthetic biology innovation ecosystems across Europe, focusing on the differences and commonalities between EU and non-EU countries. This study, conducted from June to October 2023, aims to understand the factors driving success and identifying challenges within these ecosystems. Methods: A comprehensive survey was disseminated among a diverse group of stakeholders, including policymakers, academic scholars, industry experts, and non-profit organizations. The methodology centered on a SWOT (Strengths, Weaknesses, Opportunities, Threats) analysis to thoroughly evaluate each ecosystem. Results: Preliminary findings indicate a broad spectrum of innovation capabilities, with certain ecosystems showcasing strong industry-academia collaborations and others grappling with resource and infrastructure limitations. The project's full results, focusing on specific strengths, weaknesses, opportunities, and threats identified across different ecosystems, will be detailed in the SynCellEU 2024 conference presentation. Conclusions: The SYNBEE project underscores the diversity of Europe's synthetic biology innovation ecosystems. It points towards the need for targeted strategies that bolster strong ecosystems and support emerging ones, emphasizing regulatory harmonization, increased funding avenues, and enhanced cross-sector collaboration. By addressing these areas, the project advocates for a strategic approach to solidify Europe's leadership in synthetic biology, fostering a supportive environment for sustainable innovation and growth.

*Speaker

Troubleshooting an Affordable Cell-Free Protein synthesis (CFPS) System

Charlie Newell * [1], Michael Booth

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Synthetic cells engineered to express genes can respond to environmental cues, regulate their behaviour, and communicate intercellularly to facilitate biomedical and biotechnological applications ranging from drug delivery to microbiome engineering. However, this involves the encapsulation of commercially available CFPS systems, which are generally expensive and lack customisation. OnePot Protein synthesis Using Recombinant Elements (PURE) is a cheaper and more tailorable alternative, making synthetic cell synthesis more accessible and easily specialised. OnePot PURE is prepared through co-purifying 36 proteins encoded across two plasmid types which can only be purchased from addgene. Sequencing the addgene plasmids revealed that one of the plasmid types is highly prone to mutation in the promoter region, resulting in little or no expression. Using a combination of colony polymerase chain reaction (PCR) screening and site-directed mutagenesis, we have repaired the plasmids and transformed them into a stable bacterial strain preventing future mutation and enabling complete formulation of the system. Addgene have been made aware of how to resolve this issue, which should aid the community in exploiting OnePot PURE for future cell-free and synthetic cell applications.

*Speaker

Artificial cells to study focal adhesion assembly and mechanosensing

Audrey Ntadambanya * [1], Julien Pernier [2], Susumu Kimihiro [3], Igor L. Medintz [3], A. Klymchenko [4], I. Le Potier [5], Christophe Le Clainche [1], Marcelina Cardoso Dos Santos [1]

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Transmission of force between the cell and its environment is essential for the cell to properly accomplish adhesion and migration processes required for tissue building and repair. Transmission of force across the cell membrane is facilitated by focal adhesions (FAs), which are highly organised, self-assembled microstructures. To resolve macromolecular interactions on membrane-protein interface, we are developing **"3D in vitro models".** They are based on Giant Unilamellar Vesicles (GUVs) mimicking phospholipidic cell membrane and interacting mechanosensors (specialised proteins capable of sensing cytoskeletal tension) and cytoskeleton proteins. The main advantage of these artificial cells is that they are less complex than cells and can be highly controlled. This eases the understanding of the adhesion machinery assembly and mechanosensing mechanism. In these biomimetic models, the interaction between Talin (main mechanosensor) and the membrane is quantified using Förster Resonance Energy Transfer (FRET). The FRET nanosensor is composed of Quantum Dot (QD) donor and a dye acceptor. QD-based nanosensors has their ability to measure distances greater than those achievable using conventional FRET biosensors(based on a couple of fluorescent proteins). The entire system allowed to measure distances between the membrane and membrane-recruited Talin thanks to FRET efficiencies obtained using fluorescence spectroscopy and microscopy. Different variants of it allowed understanding its conformation according to the membrane of our model. Alphafold, a program predicting protein structures, has obtained models that are consistent with our experimental data. Changes of talin conformation with cytoskeleton tension (actin and myosin) could also be studied with this system. Thus, our bottom-up in vitro model system allowed us to understand the conformation of one of the Focal Adhesion protein but also to notice its behavior under cytoskeleton tension.

*Speaker

Developing mammalian biohybrids using synthetic cell technologies

Nina O'toole * [1,2], Yuval Elani [2], Philip Jones [1]

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[2] Chemical Engineering [Imperial College London] – United Kingdom

Biohybrids are microscale machines that repurpose biological sensing, synthesis, and motile components for specified purposes. Much biohybrids research has focused on modifying biological cells through genetic manipulation or interfacing these cells with cargo externally. To further develop novel biohybrids it's necessary to introduce cargo such as synthetic organelles into cells. Therefore, we are investigating the introduction of cargo to cells using membrane fusion. Our method uses plasmonic heating of biomembranes within an optical trap to trigger on demand membrane fusion for cell modification. Through this work we have enabled interfacing between biological and synthetic cells that in later work could be used to develop novel biohybrids for regenerative therapeutics or cellular synthesis.

*Speaker

Reprogramming the synthetic cell JCVI-Syn3B for the production of active ingredients of interest to bio-based industry

Raquel S. De Oliveira [1,2], Igor O Santos [1,2], Pedro H Bürgel [1,2], Betulia De M Souto [3], Betania F Quirino [2,3], John I Glass [4], Elibio Rech [1,2], Daniela M. De C. Bittencourt * [1,2,4]

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JCVI-Syn3B is a minimal cell that encodes the essential genes of *Mycoplasma mycoides* subspecies *capri*. Its genome has less than 500 genes, the majority of which are essential. Besides serving as a research platform to understand the universal principles of life, the cell encoded by minimal genome could be used as a safe and efficient "cell factory" for producing a wide variety of compounds. In searches for hemicellulose degrading enzymes, BGL11 a multifunctional enzyme with high β-xylosidase activity, was identified in a goat rumen metagenomic library screening. The capacity of BGL11 to hydrolyze multiple substrates may be a useful characteristic for industrial application. Although recombinant BGL11 showed activity towards different substrates, the enzyme was most effective in degrading xylobiose. Here we show that JCVI-Syn3B was capable of expressing and secreting active BGL11 (80 kDa). We cloned a synthetic *BGL11* gene that was codon optimized for mycoplasma expression into the plasmid Pmod2-loxpurolox-sp-cre. The *BGL11* gene also contained a signal peptide for protein secretion. JCVI-Syn3B cells were transformed with the Pmod2-loxpurolox-sp-cre-BGL11 plasmid and the presence of *BGL11* was confirmed by PCR. BGL11 activity in the SP4 growth media was measured using nitrophenyl-beta-D-glucopyranoside (pNPG) as substrate. The amount of pNP released was measured by reading absorbance at 405 nm. The absorbance of the samples was compared to a standard curve prepared with pNP and the activity of BGL11 produced by JCVI-Syn3BBGL11 clones was confirmed. This result shows the ability of the minimal cell to produce a functional enzyme of bio-based industry interest.

*Speaker

Large Serine Integrases: Tools for building synthetic genomes

Femi Olorunniji * [1], Phoebe Rice [2]

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A key requirement for the design of synthetic cells is the ability to modularly construct and edit DNA replicons, to precisely programme selective gene expression and to manipulate multiple sets of genes that direct cellular structure and function. To build such programmable genetic circuits, we are developing Large Serine Integrases (LSIs) and their Recombination Directionality Factors (RDF) as orthogonally-acting genome editing tools that mediate predictable, controllable, and reversible rearrangements of DNA modules. New structural and biochemical insights into LSI-RDF interactions can be applied to develop more flexible specific genome rearrangement events.

*Speaker

Light-controlled DNA segregation systems for a synthetic cell

Beatriz Orozco * [1], Yash Yawale [1]

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The transfer of genetic material between cell generations often relies on active segregation facilitated by cytoskeletal filaments. Here, we present the results of the in vitro reconstitution and preliminary liposome encapsulation of the bacterial DNA segregation system ParMRC. We have engineered the adaptor protein ParR to enable functionality upon blue light exposure, achieved by fusing ParR with the LOV2 domain of the photoreceptor protein phototropin. This approach allows for spatiotemporal control of DNA processing during the synthetic cell life cycle.

*Speaker

The Global Quest for Synthetic Cell Research: Exploring Opportunities, Challenges, and Prospects in Africa

Geoffrey Otim * [1]

[1] SynBio Africa – Uganda

The quest for synthetic cell research represents a global endeavor marked by unparalleled opportunities, intricate challenges, and promising prospects. The talk will dive into the multifaceted dimensions of synthetic cell research, offering a nuanced exploration of the scientific, technical, and ethical considerations that underpin its advancement. From the complex engineering of cellular systems to the interplay of bioethical principles, the journey towards synthetic life forms navigates uncharted territories with profound implications for human health, environmental sustainability, and beyond. At the forefront of this exploration lies the Synthetic Cell Africa Initiative, set to launch at the upcoming SynBio Africa conference in July 2024, hosted in Nairobi, Kenya. Rooted in the principles of inclusive collaboration and sustainable innovation, this initiative aims to empower local talent, foster interdisciplinary research, and foster a thriving synthetic biology ecosystem across Africa. Central to the initiative's mission is the integration of ethics, safety, and responsible governance frameworks, ensuring that scientific progress aligns with societal values and global biosecurity imperatives. Through strategic partnerships with academia, industry, regulatory bodies, and forging international partnerships, Synthetic Cell Africa charts a course towards a future where synthetic biology transcends geographical boundaries to not only advance novel scientific discovery but also to address pressing societal challenges.

*Speaker

Enzyme reconstitution into PMOXA-PDMS-PMOXA-based biomimetic vesicles

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The quest to recreate the fundamental processes of life, such as respiration and compartmentalization, in protocells heavily relies on the construction of vesicles that mimic cellular compartments. Traditionally, lipid vesicles have been the go-to building blocks for these systems, owing to their biocompatibility and bilayered structure akin to cell membranes. However, susceptibility of liposomes to oxidative damage along with their structural instability significantly limits their functionality over time. Recent advancements have highlighted block and graft co-polymer membranes as robust and stress-resistant scaffolds that can sustain enzyme activity more effectively than their lipid counterparts. Among these, polymers comprising polydimethylsiloxane (PDMS) as a hydrophobic core are favored for vesicle construction because they enable protein integration with retained function. This attribute, combined with the potential for diverse chemical modifications, allows PDMS to exhibit enhanced characteristics, making it a material of choice for designing advanced vesicular systems.
In this meeting, we report on protein insertion into various polymer and hybrid vesicles synthesized from PMOXA-PDMS-PMOXA triblock copolymer. First, we explored solubilization of these vesicles with different detergents, a prerequisite for the successful detergent-mediated enzyme reconstitutions. Next, we evaluated the insertion of cytochrome *bo3* oxidase and it's long-term stability and functionality. We also successfully scaled these proteovesicles to the micron size to examine enzyme orientation and efficiency of insertion with confocal microscopy. Finally, the retained proton transport capability of *bo3* within PMOXA-PDMS-PMOXA-based giant vesicles revealed promising applications in bioenergetics and synthetic biology.

*Speaker

Interpreting cell division in the genomically minimal cell JCVI-syn3A by applying physical models for vesicles

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The genomically minimal cell JCVI-syn3A offers a simplified model system to study cell division in the absence of a peptidoglycan cell wall. JCVI-syn3A exhibits binary fission, resulting in round cells less than one micron in diameter. Deletion of some genes of unknown function perturbs cell division, generating a fraction of large cells greater than several microns in diameter. JCVI-syn3A retains the highly conserved gene *ftsZ*, which encodes a bacterial tubulin homolog that can assemble into a constricting ring at the division site in most bacteria. Surprisingly, deletion of *ftsZ* does not produce large cells, suggesting other forces contribute to constriction during cell division in JCVI-syn3A. As a reference, physical models to describe shape transformations in vesicles can predict spontaneous constriction in the absence of a constricting ring, for certain values of the surface-area-to-volume ratio and the preferred membrane curvature. We are characterizing how FtsZ may act in the biophysical context of a membrane curved by other forces. This physical view provides a quantitative framework to compare mechanisms of cell division in top-down genomically minimal cells and bottom-up synthetic cells.

*Speaker

Bio-inspired organocatalysis in coacervate protocells

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The bottom-up assembly of functional protocells requires the integration of primitive (bio)chemical reactions within simple micro-compartments. The formation of C-C bonds in the absence of enzymes is particularly critical to the prebiotic synthesis of biomolecules, but remains highly challenging in aqueous environments. Bio-inspired *N*-heterocyclic carbenes (NHCs), rationally designed as synthetic analogues of enzyme cofactors, have recently been shown to catalyze C-C bond formation in water.(1) Our goal is to expand such catalytic reactions to model compartments. Coacervates droplets formed via liquid-liquid phase separation of oppositely charged polyions in water represent viable protocell models.(2) These membrane-free droplets spontaneously uptake biomolecules by portioning, and thus support various enzyme reactions. Given their lower polarity compared to water, coacervates can also accumulate organic molecules(3) and promote non-enzymatic reactions, such as polycondensation.(4) In this work, we develop novel bio-inspired and chemically active coacervate systems consisting of an *N*-heterocyclic carbene as the cationic species combined with various negatively charged model synthetic polymers. These coacervates function as a unique reaction medium, enhancing the efficiency and selectivity of model C-C bond forming reactions. Overall, by illustrating how coacervates promote and guide non-enzymatic catalytic reactions, our findings provide new insights into the chemical emergence of complex molecules within simple protocells.

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*Speaker

Expression of an ftsZ-ftsA operon drives constriction of synthetic cells

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The Z-ring is considered the main orchestrator in cytokinesis and has been proposed as a minimal system to drive division of synthetic cells. Cell-free expression of primary Z-ring components, FtsZ and FtsA, has resulted on membrane remodelling but has failed to drive full liposome constriction nor division. Alternatively, formation of membrane necks and budding vesicles has been achieved with expressed FtsA, while FtsZ was provided in a purified form. Herein, we report on a fully gene-encoded FtsA-FtsZ protein system capable of driving drastic liposome constriction, resulting in dumbbell-like morphologies without the need of crowding agents or external stimuli. Furthermore, we provide evidence on how the strict requirement of high FtsZ:FtsA ratios described *in vivo* determines the successful reconstitution of the Z-ring in liposomes. We then apply this information on the construction of a single DNA template expressing functional FtsZ:FtsA ratios through an operon design. Our results demonstrate the relevance of operon arrays as a tool to effectively regulate the expression and activity of complex protein systems in a synthetic cell. We are now integrating other genetic modules to the *ftsZ-ftsA* minigenome, scaling up the biological complexity towards an autonomously growing and dividing synthetic cell.

*Speaker

Origins of biological information in a bottom up physiochemical protocell

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We show how selection between combinatorial co-factors can result in the origin of biological information in a simple protocellular system, if the co-factor modulates an energy transduction process that turns resources into protocellular building blocks. Further, we show that the transition from nonliving to living matter is discontinuous in protocellular biomass if the free parameter is the protocellular net growth rate. Further, amplification, selection, and evolution of co-factors can only occur once a co-factor is randomly 'discovered' that operates above this critical discontinuous threshold. Thus, this critical point is also the onset of Darwinian evolution.
We have previously experimentally demonstrated that a combinatorial co-factor (including 8-oxo-guanine) and the energy transducer (Ru2+)bpy3 anchored to a fatty acid vesicle surface can transform resources (picolinieum ester and protected DNA oligomers) into building blocks (decanoic acid and functional DNA oligomers)(DeClue et al., 2009)(Maurer et al., 2011)(Cape et al., 2012). This proto-metabolism enables the vesicle container to grow and divide (Albertsen et al., 2014) as well as DNA oligomers to ligate into a full DNA strand. In simulation we demonstrate that anchored co-factor replication is possible based on lesion induced DNA amplification (LIDA) without the use of enzymes (Bournebush et al., 2020)(Engelhardt et al. 2020)(Thomsen 2022)(Tuccio 2023).

Further, we demonstrate in simulation that 8-oxo-guanine integrated within a DNA duplex can act as an electron donor for the (Ru2+)bpy3 energy transducer due to internal DNA charge (hole) transfer properties (Thomsen, 2022), which are sequence dependent. Our simulations also indicate that the (2D) surface anchoring of the involved molecular complexes speeds up the reaction rates compared to reactions in bulk (3D) although crowding factors also impact the reaction rates (Tuccio, 2023). Thus, the co-factor sequence/composition can be interpreted as primitive biological (functional) information when selection from a combinatorial set of co-factors is possible.

*Speaker

Synthetic Cells with Integrated DNA Self-Replication and Membrane Biosynthesis

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The construction of synthetic life involves the assembly of an elementary cellular entity, designed to converge essential biological processes. While previous studies have provided valuable insights into individual biological mechanisms, they have not yet addressed the challenges that arise from the implementation of diverse cellular processes into a functioning whole. Here, we integrate three essential modules within liposome compartments: gene expression, DNA self-replication (DNArep), and membrane synthesis via phospholipid production (PLsyn). Both DNArep and PLsyn modules were expressed from a synthetic genome (*DNArep-PLsyn*) using the 'protein synthesis using recombinant elements' (PURE) system. Module compatibility, with minimal interference from substrate or cofactor crosstalk, was demonstrated under various conditions using flow cytometry and high-content fluorescence imaging. However, co-expression of *DNArep-PLsyn* decreases the overall occurrence of liposomes with an active PLsyn module and reduces the yield of self-replicating DNA. We further discuss potential optimization strategies – in particular in vitro Darwinian evolution – to accelerate module integration for engineering synthetic cells. Moreover, this minimal integrated cell-free system provides a platform to understand how cellular processes are coupled at both genetic and metabolic levels.

*Speaker

A 3D biochip for 2D large-scale-integration of genetically programmable artificial cells induces phase waves

Joshua Ricouvier * [1], Vincent Noireaux , Roy Bar-Ziv [1]

[1] Weizmann Institute of Science – Israel

The on-chip large-scale-integration of genetically programmed artificial cells capable of exhibiting collective modes is an important goal for fundamental research and technology. Here, we report a 3D biochip enabling assembly of a 2D layout of 1024 monolithic DNA compartments as artificial cells on a 5-millimeter square silicon chip. Homeostatic cell-free protein synthesis reactions driven by genetic circuits occur inside the compartments. We created a reaction-diffusion system with a 30x30 square lattice of artificial cells interconnected by thin capillaries for diffusion of products. Driving the system by a genetic oscillator revealed emergent collective modes of synchrony and propagating phase waves in 2D, with dynamics controlled by geometry. This demonstrates a class of nonequilibrium autonomous systems, where chemical energy consumed to make proteins induces 2D collective patterns of gene expression on multicellular scales, with applications in biological computing, sensing, and materials synthesis.

*Speaker

The role of membrane transport and confinement in regulating the feedback mechanisms of enzyme reactions in artificial organelles

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[4] School of Biology [Leeds] – United Kingdom

Feedback is ubiquitous within biological systems, extending over all aspects of cellular function, from heartbeats to apoptosis to cell division. The existence of feedback in cells is dependent on non-linear temporal and spatial dynamics. Feedback is central to cell survival, as without communication and regulation, a cell is unable to adapt to its environment. The complexity of feedback systems in nature is challenging to study *in vivo*, motivating many to replicate processes in biomimetic systems, such as artificial cells. Such systems enable us to explore the design principles of dynamic non-linear behaviour, developing a comprehensive understanding of chemical self-organisation utilized in cells.
A simplistic biological feedback system can be generated using a single pH-dependent enzyme that catalyses the production or consumption of H+. For example, the enzyme urease has a pH-dependent activity and catalyses a reaction that increases local pH. Therefore, there is a base-catalysed feedback mechanism that influences the reaction rate.

Combining experimental and numerical simulation, we engineer synthetic organelles encapsulating the urease enzyme. We focus on the roles of confinement and membrane transport in regulating the non-linear dynamics of the enzyme reaction. Understanding the influence of transport is fundamental to how spatiotemporal control is upheld in cells. Using our synthetic organelles, we demonstrate how selective permeability and confinement can have a decisive effect on feedback.
Additionally, the non-linear dynamics of pH-feedback enzyme systems has emerged to have promising applications in drug release, biosensors, nanomotors and hydrogel formation. Increasing understanding of how confinement and permeability can impact the reaction dynamics will be crucial to these applications.

*Speaker

pH-responsive membranes for cell-inspired GUV-based microrobots

Elisa Roberti * [1], Dario Cecchi [1], Gaia Petrucci [1], Eugenia De Remigis [1], Francesco Bianciardi [1], Michele Ibrahimi [1], Stefano Palagi [1]

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Microrobots, untethered and mobile robotic devices with micron-to-millimeter size, have the potential to navigate confined spaces within the human body, which makes them particularly promising for diverse medical applications (e.g. drug delivery). Aiming at cell-inspired microrobots capable of autonomous movement within body tissues by sensing chemical or physical gradients, we are developing vesicles with a responsive membrane. We are thus producing Giant Unilamellar Vesicles (GUVs) by the droplet-transfer method or the gentle hydration method and studying the role of the lipid composition of the membrane on its mechanical response to external conditions. In particular, to induce changes in local curvature and deformability in response to mild pH stimuli, we add acidic-sensitive lipids like linoleic acid, oleic acid, or CHEMS to the phospholipids. To investigate the deformability of the vesicles, we load them with a ferrofluid and apply an external, uniform magnetic field to elongate them. We thus analyze the vesicles' deformation varying their membrane composition and the pH of the medium. Since the surface charge of GUVs may influence membrane's deformability and curvature, we measure the zeta potential of different phospholipid compositions in buffers with different pH values. The next steps will focus on achieving membrane polarization in response to either a chemical or a physical gradient, enabling the development of chemotactic microrobots. This study will also lead to a greater understanding of the role that certain molecules play in the mechanical and chemical properties of artificial cells, thereby providing insights into mechanisms such as budding and division of natural and synthetic membranes.

*Speaker

Phagocytic synthetic cells: non-living predators to fight bacteria

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I will introduce **Phagocytic Synthetic Cells** (PSCs) that recognize, capture, engulf and kill antibiotic-resistant bacteria without generating selection pressure for resistance recapitulating the most salient steps of phagocytosis. The PSCs are synthetic vesicles that can selectively bind to bacteria and exploit the binding energy to drive engulfment. From a thermodynamic point any vesicle can engulf a bacterium if if the adhesive energy surpasses the bending energy of vesicle's membrane. However, the high curvature intermediates present during engulfment have prevented the use of the state-of-the-art vesicles for this task. We invented a new family of biomimetic vesicles, called i-combisomes that, despite having the same flexibility of superflexible liposomes, exhibited unsurpassed ability to quantitatively engulf nano- and micro-objects including bacteria which they killed upon engulfment. This superpredatory behavior is rooted in the statistic nature of the molecular topology of their building blocks, ionically-linked comb polymers. When assembled in water, their collective behavior follows a mean-field description, smearing the heterogeneity by forcing molecules with non-zero spontaneous curvature into a flat membrane acquiring a strained conformation, with the concomitant local-mean curvature mismatch. When engulfment begins, these molecules migrate to the non-zero curvature regions and adopt their more favorable conformation, therefore reducing the kinetic barriers, a trait that membranes assembled from a single low molecular weight amphiphile cannot achieve. By the same token, a few minutes after the engulfment of an antibiotic-resistant bacterium, the close apposition of the phagosome and bacterial membranes resulted in their fusion and the death inside the i-combisome. The remarkable element of this concept is that the killing occurs inside the PSCs, separated from the environment and because it targets a highly conserved element of the pathogen, its membrane, the killing action cannot be avoided by evolution. Remarkably, the PSCs were engineered to be safe for eukaryotic cells and human organoids.

*Speaker

Engineering Genetic Tools to Control Individual Microbes and Microbiota without Antibiotic Resistance Genes at a Single Strain Level

Austin Rottinghaus [1], Matthew Amrofell [1], Steven Vo [1], Tae Seok Moon [*1]

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Microbial biocontainment is essential for engineering safe living therapeutics (1, 2). However, the genetic stability of biocontainment circuits is a challenge. Kill switches are among the most difficult circuits to maintain due to the evolution of escape mutants. We engineered two CRISPR-based, chemical- or temperature-inducible kill switches in the probiotic *Escherichia coli* Nissle and demonstrated mutationally robust biocontainment (3). In this presentation, we will discuss our machine learning-based microbiota engineering tools that are useful to manipulate microbiota and kill pathogens at a single strain level (4). Specifically, we will discuss the development and validation of a novel computational program, ssCRISPR, which designs strain-specific CRISPR guide RNAs (gRNAs) that can be utilized to modify complex consortia. As a proof of concept, we applied the program to two novel applications: the isolation of specific microbes from consortia through plasmid transformations and the removal of specific microbes from consortia through liposome-packaged CRISPR antimicrobials. Additionally, we will discuss antibiotic resistance gene-free plasmid systems that prevent antibiotic resistance spread via horizontal gene transfer (5-8). This new technology has vast implications in designing strain-specific antimicrobials and combating the growing concern of antibiotic- and bacteriocide-resistant microbes.

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*Speaker

Using DNA nanostructures for lipid membrane biophysics and bioengineering in synthetic cell science

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Biological membranes tightly regulate the spatio-temporal organisation and interactions of membrane-bound proteins to facilitate a range of pathways that are key to living organisms, from signal transduction to motility and division. Synthetic cell science aims to replicate behaviours usually observed in living matter in cell-like agents constructed from the bottom-up (1). Synthetic cells are useful model systems that are routinely applied to deepen our understanding of biological processes in isolation from physiological complexity, and they are poised to unlock promised applications in healthcare and biotechnology.
By exploiting the tools of self-assembly and chemical bio-nanotechnology, my work uses DNA molecules to build nano-scaled devices to interrogate the biophysical principles governing the spatial and temporal organisation of membrane inclusions. In this talk, I will show that bespoke amphiphilic DNA nano-devices can be applied to engineer functionality in the lipid membrane of synthetic cells (2). We apply our DNA-based toolkit to regulate membrane-hosted life-like responses, from control over the organisation and transport of cargoes across membrane surfaces (3) to membrane restructuring and fission (4). Besides fundamental contributions to our understanding of the lateral organisation and activity of inclusions in biological membranes, the development of these biomimetic functionalities paves the way for next-generation sensing, communication, and division pathways in synthetic cellular systems.

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*Speaker

Exploring Synthetic Cell Division: Modeling Lipid Bilayer Membrane Dynamics

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Cell division is a fundamental process in life and also one of the major challenges in synthetic biology. While numerous nature-inspired mechanisms have been proposed, our understanding remains limited.
My aim is to model synthetic cell division in silico, with a focus on understanding the physics of lipid bilayer membranes during the process. In silico experiments can deal with spatio-temporal scales inaccessible to wet lab techniques and they can be used to test the validity of theoretical models.

By employing coarse-grained molecular dynamics simulations, I capture essential physical properties such as surface tension, bending rigidity, and thermal fluctuations response. I successfully reproduced membrane reshaping under various conditions, including osmotic shock and protein-induced curvature change.
Future research will involve modeling Dynamin as a single-protein machinery to perform cell division, in collaboration with experimentalists.

*Speaker

Resurrecting ancestral membranes in minimal living cells

James Saenz * [1], Nataliya Safronova [1], Isaac Justice [1], Ha Ngoc Anh Nguyen [1], Edward Lyman [2], Liam Sharp [2], Lisa Junghans [1]

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All cells are encapsulated by a lipid membrane which manages the relationship between life and its environment. Looking across the domains of life, we find a staggering diversity in the lipid composition of biological membranes. Since lipids determine the physical properties of membranes, which in turn influence their function, the evolution of membrane property and function is, at least indirectly, encoded in the diversification of lipid complexity. However, the principles required to account for the diverse range of lipid chemistries required for life have not been elucidated. To address this challenge we have developed tuneable living membrane systems to understand how life employs the collective properties of lipids to build responsive organizational interfaces between cells and their environment. These systems are based on mycoplasmas, which due to their dependence on exogenous lipids, gives us the ability to tune their lipidome composition. For this presentation, I will report on our insights from recent advances that allow us to tune lipidome composition in the Minimal Cell (JCVI-Syn3) from fewer than 10 to more than 100 lipid species. Through this approach we reveal that cells can survive on as few as two lipids, and that lipidome complexity has profound consequences for cellular growth and robustness, and metabolic efficiency. By introducing ancient sterol analogues (hopanoids), we explore how the evolution of sterol structure could have directed the evolution of lipidome composition during the emergence of sterol-rich eukaryotic membranes. Further, we observe profound effects resulting from subtle changes in lipid structure, such as the lipid chirality that distinguishes Archaea from the rest of life. By resurrecting ancestral lipidomes, we aim to reveal the conserved features of modern membranes and introduce a new paradigm for understanding why life needs so many lipids.

*Speaker

Dynamic Size Regulation and Oscillatory Behavior in Synthetic Cellular Compartments

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Cells are highly hierarchical structures sustained by an external energy input. To mimic this, we utilized a chemical reaction to induce phase separation through complex coacervation within a confined space where the fuel – the energy input – comes from outside the synthetic cell. This approach allowed us to study how out-of-equilibrium droplets can regulate both their size and spatial organization.

We present experimental and theoretical evidence supporting that a population of droplets can evolve to a uniform stable size provided that these droplets are regulated by a chemical reaction cycle, and fusion is hindered. Our investigation includes the impact of diverse variables, including the concentration of fuel, precursor, as well as other physicochemical parameters, on the size of these droplets. Additionally, we demonstrate the applicability of this mechanism under confined conditions within a cell-like size range.

Remarkably, our findings unveil a novel aspect of synthetic cell behavior. When the size control mechanism is active but fusion between droplets is unhindered, an oscillatory pattern emerges within the confined environment. This intriguing behavior is characterized by small droplets nucleating at the top of the container, sedimenting to the bottom where fusion occurs. Since fused droplets exceed their stable size, they expel material, thus initiating a cyclic process. Furthermore, the oscillatory pattern was also successfully described theoretically.

This research not only contributes to our understanding of dynamic size regulation within synthetic cells but also reveals the emergence of self-sustaining oscillatory behavior within confined compartments. Such insights have profound implications for the design and engineering of synthetic cellular systems, providing a platform for further exploration of compartmentalized, dynamic processes mimicking cellular behaviors.

*Speaker

Evolutionary integration of synthetic cell modules using a DNA self-replicator

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The development of a synthetic cell requires the functional integration of key biological modules. This step has been challenged by the loss of protein expression efficiency upon incorporation of genes and the poor compatibility between some of the modules. We propose to use an integrative evolutionary approach based on the coupling of a protein or module of interest to DNA self-replication. The seed replicator consists of a two-gene linear DNA encoding for the phi29 DNA polymerase and terminal protein, flanked by origins of replication (1). A self-selection mechanism is established, whereby the activity of the gene product is coupled to DNA replication through a feedback loop involving a biosensing element. While replication is hindered when the gene product is inactive, expression of functional enzymes allows DNA replication to proceed, driving the evolution process.

As a proof of concept, we introduced the *lacZ* gene into the two-gene DNA replicator, as well as a *lac* operator site upstream each of the phi29 genes. Preliminary results show that the enzymatic conversion of lactose to allolactose by the *lacZ* gene product, b-galactosidase, relieves transcriptional repression induced by LacI, enabling DNA self-replication. This reaction network can be seen as a synthetic form of interdependence between information propagation (DNA replication) and metabolism (lactose conversion). Rounds of evolution will be applied in liposomes to select for enhanced LacZ variants that lead to improved allolactose production and thereby higher levels of DNA template. This method circumvents the constraints of variant screening for *in vitro* evolution and mimics the principles of Darwinian selection. We are now designing more complex genomes for the sequential integration of synthetic cell modules via evolutionary self-improvement.

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*Speaker

Orthogonal Communication Networks in Synthetic Tissue

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Cells in multicellular organisms build dynamic networks for intercellular communication and cooperation, enabling many of the higher-order capabilities in tissue. Emulating such sophisticated behavior in synthetic materials has important applications in tissue engineering and next-generation computing. To this end, liposome-based synthetic cells (SC) have emerged as an attractive building block for synthetic tissue owing to their tunable and biomimetic properties. However, fabricating controlled and responsive networks in these lipid bilayer-based SCs has been challenging as signaling molecules must transit through two consecutive membranes between the so-called sender and receiver SCs. Here, we address this obstacle by reconstituting engineered connexon nanopores into SC membranes. In mammalian cells, a class of membrane proteins called connexins self-associate to form hexameric connexons that release cytoplasmic contents to the extracellular space and to neighboring cells. We show direct SC-to-SC communication by pairing connexons on apposing synthetic cells to form connexin-dependent channels. We find that formation of these channels is enhanced by adhesive molecules and report on optimum adhesive conditions for maximum inter-SC transfer. We also demonstrate orthogonal SC communication using different connexin isoforms: connexin43 (Cx43) and connexin32 (Cx32). To allow for user-defined communication between SCs, we re-engineer Cx43 and Cx32 connexon assemblies to be sensitive to two orthogonal proteases. We focus on light as a trigger to enable rapid activation inside SCs. To accomplish this, we cage orthogonal proteases in UV- and NIR-sensitive liposomes. When encapsulated inside SCs, these liposomes allow for responsive assembly of active Cx43 and Cx32 channels. By combining multiple connexins and liposomes in a SC assembly, we demonstrate responsive, orthogonal signal transfer in SCs, an important step toward synthetic communication networks.

*Speaker

FcRn targeted-artificial intestinal L cells as bioengineering oral delivery systems to treat diabetes

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Type 2 Diabetes Mellitus (T2DM) is one of the most common metabolic disorders characterized by hyperglycaemia, insulin resistance, and relative insulin deficiency. Current pharmacological actions mainly include administration of drugs to control blood glucose levels. However, the invasive and painful administration routes of antidiabetic drugs frequently dictate poor patient compliance, contributing to a leading cause of mortality and morbidity. Glucagon-like peptide 1 (GLP-1) is an incretin hormone which is released from enteroendocrine L-cells located in the gut that acts to augment postprandial insulin levels in healthy patients. In T2DM patients, this incretin effect is markedly diminished. To maintain patient compliance, oral administration of GLP1-producing artificial cells represents a promising strategy to restore tight glycemic control. In this project we propose the development of a system that mimics intestinal L cells function to produce GLP-1 peptide and is surface modified with moieties to target intestinal neonatal Fc receptor (FcRn) for enhanced transport across the intestinal epithelial. The FcRn is an intestinal receptor-mediated pathway for drugs and drug carriers, present in apical region of enterocytes. The novel peptide-producing particles will be constructed by encapsulating *E. coli*-based cell-free protein synthesis (CFPS) system combined with a plasmid encoding to GLP-1 peptide inside POPC/DSPE-PEG(2000)-Mal/Chol vesicles by water-in-oil transfer method. The FcRn-targeted ligand, peptide CQRFVTGHFGGLYPANG, was already conjugated with DSPE-PEG(2000)-Maleimide by Mal-thiol chemistry. Permeability of functionalized SCs will be assessed *in vitro* using 3D intestinal models and the glucoregulatory effects will be evaluated on INS-1E β cells. Therapeutic potential will also be assessed *in vivo* by performing biodistribution, safety and functional studies in diabetic animals.

*Speaker

Communication between DNA Protocells and Living Cells

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Designing and building protocells from scratch by combining functional and structural parts with life-like properties provides a versatile approach to fabricating cell-mimics. In addition, engineering chemical communication between protocell models and living organisms via signal processing has evolved into an appealing research direction in the field of synthetic biology and bioengineering, which can provide emergent properties and creative applications. DNA-based condensates are recognized as excellent biomacromolecule compartments for protocell mimicking, because they are capable of programming structure formation and rationally designed reaction networks. Via mimicking the biological complexity, downstream functional responses of DNA protocells such as functional adaptation, mechanical activation, and structural morphogenesis of protocells in a life-like manner as well as communication and interaction with living cells, can be realized in a facile manner. We aim to demonstrate the ability of DNA protocells as a platform that can modulate cellular communication, sense and process stimuli originating from living cells. By integration of RGD motifs into DNA mechanosensors, protocells will be empowered to sense force exerted by living cells. Finally, we will connect mechanosensing with DNA-based downstream reactions for triggering protocells' response, thus establishing adaptive and interactive communication between DNA protocells and living cells.

*Speaker

Droplet-based synthetic cells and lymphatic tissues for expansion of therapy-relevant T cells

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The advancement of effective adoptive immunotherapy heavily relies on *ex vivo* expansion of T cells. A primary challenge in this domain is to expand T cells with specific, therapy-beneficial phenotypes, such as effector, regulatory or memory cells. This has been hindered by a lack of technologies that closely replicate the natural expansion process of T cells by antigen-presenting cells (APCs) within lymph nodes, where the required environmental cues to drive directed and specific T cell expansion are provided. To address this, we introduce a synthetic cell technology for directed T cell expansion. This technology utilizes dispersed liquid-liquid phase-separated droplet-supported lipid bilayers (dsLBs), designed as artificial antigen-presenting cells (aAPCs). These aAPCs have adjustable biochemical and biophysical properties, enabling directed *in vitro* T cell activation and differentiation. The dsLBs are engineered to present T cell receptor ligands and tumor-associated antigens on their surface, stimulating T cell activation. They can also secrete stimulatory cytokines and emulate key biophysical APC properties like stiffness and membrane fluidity. Furthermore, we show how they can be assembled into three-dimensional artificial tissue structures, simulating the lymph node microenvironment. I will present three key finding obtained with this synthetic tissue: Firstly, the selective expansion of IL-4/IL-10 secreting regulatory CD8+ T cells with a PD1/CTLA4 negative phenotype, which are less susceptible to immune suppression. Secondly, evidence that lateral ligand mobility can mask the differences in T cell activation typically obtained on substrates of varying stiffness. Thirdly, proof for a mechanosensitive component in bispecific Her2/CD3 T cell engager-mediated immunotherapy. Our development underscores the potential of synthetic cell technologies in synthetic immunology and represents a significant step towards integrating these advancements into pre-clinical protocols.

*Speaker

Communication within Artificial Cell Mediated Tissue Engineering

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Artificial cell-mediated tissue engineering approaches have started to gain interest to study the translational applications of synthetic cells in regenerative medicine studies. 3D bioprinting is a versatile technique for this purpose as it allows to combine artificial cells along with biomaterial inks into an engineered final product which can mimic the biological and physicochemical properties of natural tissues. In this study, a communication pathway was set up within a 3D-printed scaffold. Terpolymer stabilized amylose-coacervates as artificial cell models were embedded into gelatin based biomaterial ink formulation to fabricate artificial tissue models. The model cargo protein was sequestered by the coacervate population via a DNA-mediated uptake mechanism with a subsequently triggered release behavior within the 3D-printed scaffolds. Artificial cell containing biomaterial ink combinations were characterized by rheology studies along with fluorescence recovery after photobleaching (FRAP) studies to evaluate the diffusion behavior of the cargo molecules.

*Speaker

In vitro evolution of T7 RNA polymerase facilitated by droplet microfluidics

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Efficient ways to produce single-stranded DNA are of great interest for diverse applications in molecular biology and nanotechnology. The flagship platform to replace the phosphoramidite synthesis process is envisioned to be template-independent catalysis by terminal deoxynucleotidyl transferase, although specific conditions are needed to achieve controllable DNA synthesis. Among template-directed methods, asymmetric PCR (aPCR) is suggested as a means to synthesize long ssDNA products, although several reports indicate that the initial optimization of aPCR is not easy and different ssDNAs cannot be produced using a single protocol. Here, we suggest considering in vitro transcription (IVT) using highly processive T7 RNA polymerase (T7 RNAP) as an alternative approach for isothermal synthesis of ssDNA. To achieve that, we engineered T7 RNA polymerase to incorporate deoxynucleotide triphosphates (dNTPs) so that ssDNA is produced directly by IVT. We performed in vitro evolution employing droplet microfluidics. Briefly, E. coli cells expressing mutant T7 RNAP variants were encapsulated in droplets together with lysis and IVT reagents, as well as dNTPs and a fluorescent reporter. After the in-droplet IVT assay, fluorescence-activated droplet sorting was used to enrich the variants able to produce transcripts utilizing dNTPs. We identified mutations V783M, V783L, V689Q, and G555L as novel T7 RNAP variants leading to relaxed substrate discrimination. Transcribed chimeric oligonucleotides were tested in PCR, and the quality of amplification products as well as fidelity of oligonucleotide synthesis were assessed by NGS. We concluded that enzymatically produced chimeric DNA transcripts contain significantly fewer deletions and insertions compared to chemically synthesized counterparts and can successfully serve as PCR primers, making the evolved enzymes superior for simple and cheap one-pot synthesis of multiple chimeric DNA oligonucleotides in parallel using a plethora of premixed templates.

*Speaker

Platform integration for high-throughput functional screening applications

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Screening throughput is a common bottleneck in many research areas, including functional genomics, drug discovery, and directed evolution. High-throughput screening techniques can be classified into two main categories: (i) affinity-based screening and (ii) functional screening. The first one relies on binding assays that provide information about the affinity of a test molecule for a target binding site. Binding assays are relatively easy to establish, however, they reveal no functional activity. In contrast, functional assays show an effect triggered by the interaction of a ligand at a target binding site. Functional assays might be based on a broad range of readouts, such as cell proliferation, reporter gene expression, downstream signaling, and other effects that are a consequence of ligand binding. Screening of large cell or gene libraries based on direct activity rather than binding affinity is now a preferred strategy in many areas of research as functional assays more closely resemble the context where entities of interest are anticipated to act. Droplet sorting is the basis of high-throughput functional biological screening, yet its applicability is limited due to the technical complexity of integrating high-performance droplet analysis and manipulation systems. As a solution, the Atrandi Biosciences Styx platform enables custom droplet sorting workflows, which are necessary for the development of early-stage or complex biological therapeutics or industrially important biocatalysts.

*Speaker

Engineering Cyborg Cells as Dynamic Micromachines

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Two major paradigms are used to create bio-micromachines. The first uses materials and biomolecules to create artificial cells that mimic certain properties of living cells. The second modifies genes of living cells to enable new synthetic functions. Here, I present a new paradigm of creating hybrid material-cell entities, called Cyborg Cells, with superior functionalities to their constituents. Cyborg Cells are created by forming synthetic hydrogels inside each cell, rendering them incapable of dividing but remaining metabolically active. Thus far, we have demonstrated that these cells exist in a "Cyborg State" where they maintain key cellular functions such as protein expression, gene regulation, secretion, metabolism, and membrane fluidity while becoming unable to divide. Our Cyborg Cells also gain non-native resistance to environmental stressors, including high pH, hydrogen peroxide, apoptosis inducers, and antibiotics. The activity and functions of Cyborg Cells can also be modulated by controlling intracellular hydrogelation with different molecular compositions and sizes. In addition, we have generalized the approach to a broad range of bacterial species, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella enterica*, as well as mammalian cells, including primary mesenchymal stem cells, cancer cells, and immune cells. These Cyborg Cells show promising results in biosensing, antibacterial, anticancer, and regeneration therapy. Our research establishes a new frontier in synthetic biology by driving living cells to a "Cyborg" state. The unique characteristics of Cyborg Cells powered by a combination of synthetic biology, materials science, and bioengineering principles enable new biotechnological applications that were previously challenging.

*Speaker

Giant vesicles get energized: correlating membrane potentials to ion transport through fluorescence analysis

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Cells generate electrochemical gradients to drive a myriad of fundamental processes from nutrient uptake and ATP synthesis to neuronal transduction. To maintain these gradients, all cellular membranes carefully regulate ionic fluxes using a broad array of transport proteins. For that reason, it is also extremely difficult to untangle specific ion transport pathways and link them to membrane potential variations in live cell studies. Conversely, synthetic membrane models, such as black lipid membranes and liposomes, are free of the structural complexity of cells and thus enable to isolate particular ion transport mechanisms and study them under tightly controlled conditions. Still, there is a lack of quantitative methods for correlating ionic fluxes to electrochemical gradient buildup in membrane models. Consequently, the use of these models as a tool for unravelling the coupling between ion transport and electrochemical gradients is limited. We developed a fluorescence-based approach for resolving the dynamic variation of membrane potential in response to ionic flux across giant unilamellar vesicles (GUVs). To gain maximal control over the size and membrane composition of these micron-sized liposomes, we developed an integrated microfluidic platform that is capable of high-throughput production and purification of monodispersed GUVs. By combining our microfluidic platform with quantitative fluorescence analysis, we determined the permeation rate of two biologically important electrolytes – protons(H+) and potassium ions (K+) – and were able to correlate their flux with electrochemical gradient accumulation across the lipid bilayer of single GUVs. Through applying similar analysis principles, we also determined the permeation rate of K+ across two archetypal ion channels, gramicidin A and outer membrane porin F (OmpF). We then showed that the translocation rate of H+ across gramicidin A is four orders of magnitude higher than that of K+ unlike in the case of OmpF where similar transport rates were evaluated for both ions.

*Speaker

Towards assembly of self-replicating protocells

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Prebiotically plausible pathways for the emergence of a self-replicating protocell have been under investigation for a while. To this end, model protocells are typically constructed by encapsulation of nucleic acids inside fatty acid vesicles. However, such protocells cannot withstand the concentrations of magnesium ions required for the function and replication of nucleic acids. Consequently, there is no plausible path for the emergence of a self-replicating protocell. Uncovering some bits of this puzzle, here we present prebiotically-plausible and stable protocells that can grow, divide and support template-directed, nonenzymatic RNA copying as the first step for primitive genome replication. Protocells built this way may serve as a chassis for prebiotic reactions, autocatalytic networks, or cell-free enzymatic systems, coupling rudimentary forms of metabolism to heredity. Therefore, a forward-looking agenda is now plausible for investigating the conditions for the emergence of natural selection and Darwinian evolution.

*Speaker

Towards the bottom-up assembly of synthetic cells on a chip

Bert Van Herck * [1], Gijsje Koenderink [1], Cees Dekker [1]

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Despite our profound understanding of the molecular components that make up living cells, it still remains mainly unknown how these molecules cooperate to define life. Therefore, constructing a synthetic cell using a bottom-up approach, where non-living materials are organized into systems that mimic the functions of living cells, is emerging as a pioneering direction toward elucidating the intricate working mechanism of living cells. This approach not only holds the potential to revolutionize our understanding of biological systems, but also paves the way for the development of numerous applications. A key feature of a living cell is a functional cell cycle. Recent developments led to the establishment of several functional modules mimicking a specific aspect of a living cell. Now the time has come for the stepwise integration of these modules into a synthetic cell cycle. Our objective is to develop a synthetic life cycle for liposomes, focusing particularly on two fundamental characteristics of living systems: growth and division. We intend to do this on a microfluidic device, where the liposomes are produced, grow, divide, and are recirculated to repeat this cycle. More specifically, we employ a microfluidic technique termed octanol-assisted liposome assembly (OLA) that was previously established in our lab to produce cell-sized liposomes on a chip. Further, we are exploring liposome fusion as a method to grow liposomes. We set up a Förster resonance energy transfer (FRET) assay to asses lipid mixing as a measure of liposome fusion. Using this assay, we demonstrate that the use of oppositely charged membranes does induce fusion between cell-sized liposomes (5–20 μm) and small feeder liposomes (200 nm).

*Speaker

Integrating membrane synthesis and division in gene-expressing liposomes.

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Building an autonomously growing and dividing synthetic cell is one of the major scientific and bio-engineering challenges of this century. While we understand and can engineer simple biological modules from molecular components, we do not yet grasp how multiple modules collectively interact to produce an integrated 'living' cell. It is still technically challenging to co-reconstitute multiple functions in the first place, and few attempts exist for module integration. In this work, we aim at integrating two fundamental cellular processes, membrane synthesis and division, inside gene-expressing liposomes. We have genetically encoded the bacterial division proteins FtsZ (fused to mVenus) and FtsA on a single DNA template (div-DNA) whose expression in PURE system results in liposome constriction. Membrane synthesis is encoded on a separate DNA template (lip-DNA) containing the four genes of the truncated Kennedy pathway required for the synthesis of phosphatidylserine. Integration of these two modules was explored by co-encapsulating the DNA templates in different stoichiometries. Relatively low concentrations of lip-DNA favoured the occurrence of liposomes producing both phosphatidylserine and FtsZ-mVenus. Under this condition, we identified the first instances of constriction and membrane synthesis. However, localisation of FtsZ-mVenus in the lumen without constriction was more common. We attempted to increase the occurrence of liposomes exhibiting the two functional modules by varying the initial molecular composition and improving lipid precursor availability. These results have given us insights on how to combine both genetic modules on a single DNA template, which will enable the coupling of liposome growth and division through evolutionary optimization.

*Speaker

Functional Membrane Protein Integration in Synthetic Cells

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A critical aspect for the bottom-up development of a synthetic cell is the functional insertion of newly synthesized membrane proteins into the membrane. In living cells, this process is mediated by the translocon, a heterodimeric integral membrane protein complex. In bacteria this complex is termed SecYEG, and binds translating ribosomes to co-translationally insert growing polypeptide chains into the membrane. Although *in vitro*, membrane protein insertion can occur spontaneously, in synthetic cells this process likely needs to be supported by the translocon to efficiently couple protein synthesis to functional membrane protein insertion, and avoid membrane protein aggregation. As a model for functional membrane protein biogenesis, we have chosen the well-documented and versatile phosphoenolpyruvate (PEP):phosphotransferase system (PTS). This system couples the transmembrane transport of sugars to their phosphorylation. Membrane-integration of mannitol permease (MtlA) is dependent on the translocon, occurs co-translationally and does not require the activity of SecA, a motor protein that is typically involved in protein translocation (Den Uijl et al., 2024). We have developed a method to analyse the functional insertion and folding of the permease domain (MtlA), a polytopic integral membrane protein. Here, purified MtlA either reconstituted or in present in detergent micelles, is supplemented with the other components of the PTS system as well a mannitol-phosphate-dehydrogenase. Mannitol permease activity, i.e., formation of mannitol-1-phosphate, can be demonstrated through NADH formation detected using a fluorometer. Further work combines this assay with *in vitro* protein synthesis and SecYEG-assisted insertion.

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*Speaker

Evolution of synthetic cells with complex phenotypes

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Synthetic cells provide a minimal model system to study fundamental principles of life. One approach to the construction of synthetic cells requires the stepwise integration of primary cellular functions, such as DNA replication, cell growth and division. We aim at accelerating the optimization and integration of these biological functions using in vitro evolution. With this methodology, gene-expressing liposomes displaying the desired phenotype are sorted from a large population of genetic variants. We are exploring different methods to identify and sort these liposomes. First, we use high-throughput fluorescence-activated cell sorting (FACS) to screen large libraries and isolate the DNA templates that promote in vitro phospholipid synthesis. Second, we are developing a microscopy-based selection method, which we integrate with real-time artificial intelligence-assisted image analysis for spatial and temporal phenotypic interrogation. This image-based technique can select liposomes based on multidimensional features, including cell shape, protein localization and dynamic behaviours. We believe that these foundational technologies will enable the directed evolution of complex synthetic genomes, which is indispensable for the construction of a minimal cell with integrated functionalities.

*Speaker

From Synthetic Cell Division to Osmolarity Sensing

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Two fundamental challenges in bottom-up synthetic biology are vesicular compartment division and encapsulated DNA segregation. Biophyical approaches leveraging phase-separation principles provide intermediate solutions.

First, we demonstrate controlled division of phase-separated giant unilamellar lipid vesicles (GUVs) by osmotic deflation. Neck scission is achieved thanks to the line tension emerging at the phase boundary between the liquid ordered and the liquid disordered phase. According to an analytical model, we demonstrate experimentally that symmetric division requires an osmolarity ratio of $\sqrt{2}$, while asymmetric division is achievable at lower ratios. Suitable osmolarity changes were achieved by water evaporation, enzymatic sucrose decomposition, or light-induced uncaging of photocaged compounds. Furthermore, phase-separated vesicles are re-grown by feeding with single-phased SUVs using programmable DNA tags(1).

While initially driven by curiosity, the division of GUVs reveals potential for technological applications. Our quantitative description of GUV shape during the division process allows us to measure real-time changes in osmolarity in solution surrounding, effectively transforming the dividing vesicles into osmolarity sensors, compatible with live cell microscopy(2).

In parallel, we employ DNA Y-motif droplets for DNA segregation within GUVs. Liquid-liquid phase separation yields DNA droplets, with full spatial segregation facilitated by photocleavage of linking components. Confinement influences segregation dynamics, modulated by solution ionic strength and nucleobase sequences, as validated experimentally and theoretically(3).

Our work thus exemplifies how research on synthetic cell division can lead to unexpected technological applications of synthetic cell research.

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*Speaker

Engineering of compartments in coacervate-based artificial cells via controlled nucleation of the polymer building blocks

Madelief Verwiel * [1], Nadia Erkamp [2], Daoyuan Qian [2], Tomas Sneideris [2], Frans Spaepen [3], David Weitz [3], Tuomas Knowles [2], Jan Van Hest [1]

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Compartmentalization is one of the hallmarks of dynamic living systems. This includes the cell being a compartment by itself, but also the subcellular compartments found in cells, such as (membraneless) organelles which can have substructures themselves. In order to understand, mimic, or reproduce life it is useful to incorporate this hallmark into synthetic cellular platforms. In this work, compartments are realized by incorporating 38nt ssDNA in our previously described amylose-based coacervates. The amyloses are either carboxymethylated or quaternized, yielding negatively chraged (Cm) and positively charged (Q) polymers respectively. Addition of the DNA results in multiphase separation yielding three phases: the dilute phase (dilute in all polymers), a DNA poor phase (rich in Cm-amylose), and a DNA rich phase (rich in DNA and Q-amylose). This multiphase separation can be tuned by changing the interaction strengths of the polymers by changing parameters as for example the DNA length and concentration, temperature, salt concentration, or amylose charge ratio. When these parameters are changed fast enough, this can lead to the nucleation of phases in one another, forming complex out-of-equilibrium architectures. These complex structures arise from dynamically arrested phase separation and can be predicted by using phase diagrams and tie lines. This work provides a detailed method for understanding and designing a range of condensate architectures. It also provides an example of specific cargo uptake in one of the condensate phases and shows increased uptake of the cargo when complex multiphase architectures are present. Access to these complex architectures will enable researchers to incorporate increasingly sophisticated compartmentalization and functionality in condensates.

*Speaker

Single Cell Model – a framework for bottom-up cell design

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Cooper-Helmstetter-Donachie cell cycle model was joined in the development of the Single Cell Modelling Framework (SCM) with the constraint-based approach for the analysis and design of the metabolism of growing bacterial cells assuming that ribosomes are the main actors in the self-reproduction processes in the composition of the cellular chassis responsible for the doubling of cells (see Abner et al 2023). It is possible to calculate all the growing cell parameters - cell size, growth rate, cell composition considering the geometry of cells, metabolic flux patterns, etc. in the growth space of cells and determine the growth space limits based on the knowledge of the structure of metabolic reactions network and molecular characteristics of enzymes and other cellular components in the SCM framework. Models of cells of different complexity, including simple cells, allow presentation and explain the peculiarities of the SCM framework, but also Genome-Wide Models that make possible modelling and analysis of actual " cells and comparing the results of calculations with the experimental data obtained with different cells have been developed. A software environment making possible development of models of different complexity containing tools for dynamic curation of the metabolic networks (Escher tool etc.), and semi-automatic transformation of Cobra models into SCM models was also developed. The software environment can be found and tested at www.singlecellmodel.com. The approach described has been validated with the data of *E. coli, Str. thermophilus* and other microorganisms.

*Speaker

Artificial cells for in vivo biomedical applications through red blood cell biomimicry

Jorik Waeterschoot * [1], Willemien Gosselé [1], Špela Lemež [1], Xavier Casadevall I Solvas [1]

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Recent progress in the development of artificial cells highlights the potential of these platforms as therapeutic agents that mimic the physiological effects of natural cells. However, for therapeutic artificial cells to succeed, these must be able to withstand in vivo conditions, such as the high shear forces experienced in the microcirculation. On the other hand, nature's designs, such as the red blood cell, can circulate for up to four months in these demanding environments. Here, we outline the key properties of red blood cells that enable their prolonged circulation and discuss potential strategies for transferring these blueprints to synthetic systems, so that the vision of generating artificial therapeutic cells can be realised.

*Speaker

Artificial methylotrophic cells via bottom-up integration of a methanol assimilation functions

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In this century, abundant methanol feedstocks have gained substantial attention as they are capable of being further biologically processed into sustainable value-added biomass and can be manufactured from renewable resources, greatly helping achieve the carbon neutrality mission. Biological conversion of methanol using microbes has challenges; while domesticating native methylotrophs or engineering synthetic methylotrophs has advanced substantially, a lack of productivity and metabolic bottlenecks, respectively, hold back progress due to innate cellular complexities. Therefore, we have devised a parallel strategy using artificial cells to overcome the limitations of the living system. Using a cell-free expression system and liposome encapsulation, such an artificial system provides a simplified and well-defined environment for unambiguous methanol assimilation, expression of required enzymes, and functionalisation. We envision this system as an alternative to speed up the development of methanol biological utilisation, which will potentially shift sugar-based bioproduction towards a sustainable methanol bioeconomy. In this presentation, I will outline the current progress on recapitulating the methanol assimilation pathway to develop artificial methylotrophic cells.

*Speaker

Protein Condensates As Synthetic Organelles To Program Cellular Functions

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A grand challenge in the field of synthetic biology is achieving predictable and programmable control of cell behavior. To do so, we must integrate facile control systems within living systems that provide modular regulatory control over subcellular processes and cell functions. Cells subcompartmentalize biochemical processes to enhance the rate and fidelity of these reactions. Numerous regulatory systems are partitioned to distinct membraneless compartments including nucleoli, nuclear speckles, stress granules and germ granules. These biomolecular condensates are heterotypic assemblies often comprised of intrinsically disordered proteins (IDPs) and RNAs, or other multivalent polymers. Designer condensates, constructed from single proteins and which display controllable size, cargo capacity and controlled release functions promise a new generation of control systems in living and protocell systems. Using model disordered polypeptides, we have developed synthetic organelle platforms that can be synthesized in vitro or genetically encoded in cells. They function either in an 'insulator mode' for dynamic sequestration-release of native signaling components or in a 'bioreactor mode' to co-localize enzymes to enhance reaction rates. We determined the sequence rules for homotypic assembly of the germ granule protein LAF-1, and re-engineered this protein to assembly and disassemble in response to enzymatic, optical and thermal stimuli. We have biophysically characterized these coacervates in vitro, in protocells and in living cells and achieved selective concentration of targets to these designer compartments. Recently we determined the selectivity of IDP coacervation and identified pairs of polypeptides capable of forming discrete condensed phases in vitro and in living cells. Additionally, we can now deliver these coacervates to cells non-genetically, where they serve as signaling hubs or chemical compartments. Designer synthetic organelles hold great promise for bioscience both as linkable modules to create synthetic life from the bottom-up and as embedded protein circuits for synthetic biologists to program cell decision-making.

*Speaker

Lipopolymer hybrid vesicles as a platform for nanoreactors

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Nanoreactors are nano- to micro-sized confined spaces that encapsulate a catalyst to compartmentalize chemical reactions. Such confined spaces can often be created with phospholipid vesicles. Mixing phospholipids with amphiphilic copolymers has been shown to enhance the chemical and physical stability of these vesicles while retaining biocompatibility with membrane proteins. This project aims to integrate membrane transporter proteins into lipopolymer hybrid membranes to enable the specific transport of negatively charged molecules, such as fumarate and succinate, across the membrane. Furthermore, the potential of hybrid vesicles as nanoreactors is explored by encapsulating a fumarate reductase, creating a nanocompartment that facilitates the reduction of fumarate to succinate. Several C4-dicarboxylate transporters from *Escherichia coli* and *Shewanella oneidensis* have been recombinantly expressed and purified. Among these, a transporter specific for *Shewanella* was found. The transporter shares a similar structure to the dicarboxylate transporter DctA from *Escherichia coli* (AlphaFold prediction) and has been shown to bind C4-dicarboxylates such as fumarate and succinate.

*Speaker

Self-growing protocell droplet formation in aqueous two-phase system induced by internal nucleic acid polymerization

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The bottom-up reconstitution of an autonomous artificial cell is a great challenge in synthetic biology. Artificial cells with essential characteristics of natural cells such as gene replication, gene expression, energy regeneration, or division/fission have already been reported. However, building a self-growing compartment remains a challenge, which is a milestone toward creating an autonomous artificial cell. Recently, we found that DNA lowered the threshold concentration of aqueous two-phase system (ATPS) of polyethylene glycol (PEG) and dextran (DEX), which are enriched in the DEX-rich phase. Also, we found that the many numbers of small DEX-rich droplets have emerged after the DNA amplification in the miscible mixture of DEX and PEG slightly below the threshold concentration. From these results, we hypothesized that internal DNA amplification would induce the DEX-rich droplet growth. We tested rolling circle amplification (RCA) using phi29 DNA polymerase within DEX-rich droplets, and they exhibit clear volume expansion. Furthermore, we introduced the transcription-translation (TXTL) coupled DNA replication system into the DEX-rich droplet. We chose the phi29 virus DNA replication system, which is recursive, isothermal, and consists of only four proteins. DNA is replicated by self-encoded, de novo synthesized phi29 proteins inside the DEX-rich droplets. We successfully achieved approximately 100-fold replication of DNA, resulting in a 1.5- to 2-fold increase in the volume of the DEX-rich droplets. Our system shows the first example of artificial cell growth coupled with internal DNA replication. The incorporation of division machinery with our system would open the way for self-replicating artificial cell models.

*Speaker

ATP regeneration in PURE from pyruvate and inorganic phosphate

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The PURE cell-free system stands as a versatile platform for synthetic biology applications, offering a controlled environment for protein synthesis devoid of cellular complexities. Considering its minimal nature, integrating *de novo* metabolic pathways into the PURE system offers opportunities for rational bottom-up metabolic engineering supported by modular pathway design, efficient experimentation, and mechanistic modelling, which can eventually be applied to the bottom-up construction of complex synthetic cells. In this study, we construct a synthetic ATP regeneration pathway in PURE, originally implemented by Kim and Swartz in *E. coli* lysates. The pathway consists of three enzymes: pyruvate oxidase, acetate kinase, and catalase, which together regenerate ATP from an acetyl phosphate intermediate. As substrates, it uses inexpensive pyruvate and inorganic phosphate. We demonstrate the functional purification of the three enzymes, and the activity of the combined pathway in a homemade PURE system, which was subsequently optimised using a design of experiments approach. The pathway by itself is capable of powering the synthesis of 72 μg/ml of mCherry over 4 h. Compared to the existing creatine phosphate/creatine kinase system, which produces 130 μg/ml of mCherry over 4 h, the pathway is not an effective replacement. However, the two pathways in combination can produce 230 μg/ml of mCherry over 4 h, which suggests that implementing novel synthetic metabolic systems is a feasible strategy for improving the performance of PURE systems.

*Speaker

Light-Powered Cell-Free Transcription-Translation

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In living systems, the genetic program controls the expression of cellular components necessary to harness energy and synthesize building blocks, that are in turn required for its execution. In this project, I aim to develop a cell-free transcription-translation (TX-TL) system that is powered by ATP generated using light energy. Specifically, photosynthetically active thylakoid membrane-based energy modules (TEMs) were coupled to the protein synthesis by recombinant elements (PURE) system. To this end, TEMs and a PURE system without its energy-regeneration system (ΔE-PURE) were first independently established, characterized, and optimized. Next, undesired interactions between the two in vitro modules were identified by performing extensive cross-evaluations of system components. After testing various approaches to resolve these incompatibilities, TEMs and ΔE-PURE were combined together in an attempt to demonstrate light-driven cell-free TX-TL. This work represents significant strides towards the realization of a light-powered metabolic and genetic linked in vitro network, which would serve as the basis for the development of more sophisticated life-like systems that show emergent properties in vitro.

*Speaker

Probing the Transformation from Membrane-less Coacervates to Membranized Coacervates and Giant Vesicles

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Compartmentalization and location of enzymes play an important role in the design and fabrication of biomimetic organelles and cells for carrying out spatiotemporal, dynamic and feedback-controlled enzymatic reactions (e.g. imitation of pH homeostasis in protocells or macrophage-/lysosome-like digestion). Thus, membrane characteristics are deciding to being successful in organelle and protocell functions which is a main focus in our working group (Adv. Sci. 2023, 10, 2207214; Adv. Funct. Mater. 2023, 2306904.) Moreover, the recent progress in the membranization of membrane-less coacervates and the coacervates-vesicles transformation paves the way for the fabrication of protocells with complex structures and functions.
However, a challenge lies in crossing the elusive "transformation boundary" from membranized coacervates to vesicles. Herein, we develop a synthetic approach for carrying out a continuous protocell transformation that can induce membrane-less coacervates into membranized coacervates and, ultimately, to giant hybrid vesicles. This transformation is initiated by the gradual and continuous addition of an anionic triblock copolymer (terpolymer) to membrane-less coacervates droplets, thereby leading to spontaneous membranization of terpolymer nanoparticles at the coacervate surface, disassembly of the coacervate phase, and the redistribution of coacervate components in the membrane architecture. Moreover, the protocells in this transformation system show different structural characteristics and molecular permeability, thereby providing the opportunities for the integration of diverse (biological) components and functions. Notably, multiphase coacervates are incorporated into this system and membranized coacervates/giant vesicles featuring distinct diffusible compartments are established.
In short, our study shows a novel avenue for the development of multi-morphological protocells transformation system with complex (membrane) architectures and introduces a new approach toward synthetic cells with morphological re-configuration (J. Am. Chem. Soc. 2024, under review). This approach reveals a strategy for inducing complex behavior in potential adaptive and dynamic microsystems that can therefore serve as a versatile tool in cytomimetic engineering and synthetic biology.

*Speaker

Magnetically Controlled-Activation of Biochemical Synthesis within Synthetic Cells

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Synthetic cells can be constructed from diverse molecular components, without the design constraints found when modifying biological systems. This can be exploited to generate cells with abiotic components creating functionalities absent in biology. One example is magnetic responsiveness which is absent from existing synthetic cell designs. This is a critical oversight, as magnetic fields are uniquely bio-orthogonal, non-invasive, and highly penetrative. We have addressed this by producing synthetic magneto-responsive organelles, coupling thermoresponsive membranes with hyperthermic Fe3O4 nanoparticles to modulate encapsulated biochemical processes using a magnetic field. Combining these systems enables synthetic cell microreactors to be built using a nested vesicle design, which responds to an alternating magnetic field through in-situ enzymatic catalysis. We have also demonstrated the modulation of biochemical reactions by exploiting different lipid compositions and magnetic field strengths. This platform will unlock a wide range of applications for synthetic cells as programmable micromachines in biomedicine and biotechnology.

*Speaker

Reconstructing Archaeal Divisomes for Minimal Cells

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The ESCRT protein machinery is highly conserved across eukaryotes. In the last few years, this protein system has brought increasingly more interest because of its homology in the archaeal clade closest to the origin of modern cells. These protein complexes in mammals are responsible for many cellular processes such as cytokinetic abscission, cargo trafficking, viral budding and many more. The ESCRT complexes are however much "simpler" in the Asgard archaea clade, with only 2 ESCRT-III homologs against 12 in humans. The metagenomic sequencing and prediction of these earlier evolved homologous proteins are thought to maintain the characteristic structure of canonical ESCRTs. We have therefore produced these synthetic archaeal ESCRTs and are exploring the potential multitude of functions in a 2-protein system by reconstituting these *in vitro*. In this poster, promising preliminary data of these protein behaviors are shown in combination with archaeal and eukaryotic lipid membranes.

*Speaker

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