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Reconstituting division in minimal cell-like systems (DIVINOCELL)

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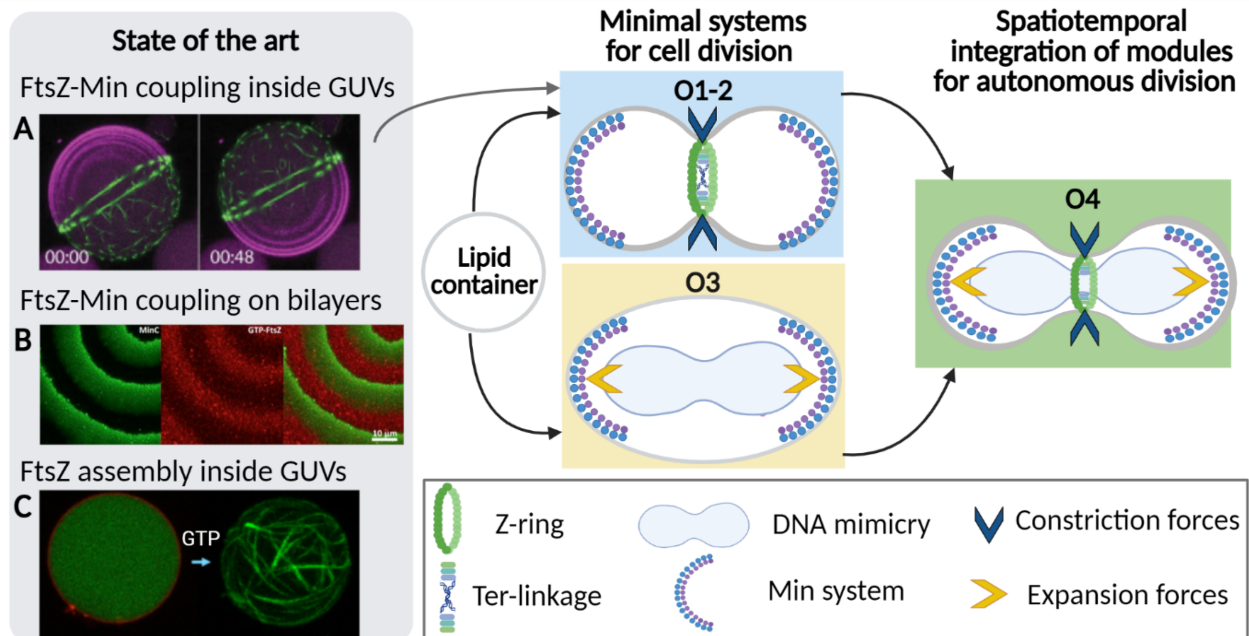
The assembly of autonomous self-replicating artificial cells from molecular components that exhibit the essential characteristics of life is one of the grand contemporary scientific challenges. It requires interdisciplinary skillsets to design and integrate biochemical modules at different levels of hierarchy. We aim to reconstitute minimal machinery for autonomous cell division, one of life's most stunning and central features. We will combine our expertise in biochemistry and biophysics of different membrane systems and protein machines to design modular reaction environments that resemble the physical appearance of a cell and its key substructures, implementing physical-chemical features essential to support cell division. Importantly, we will couple the assembly and dynamics of a division machinery consisting of a contractile ring and an expansion module perpendicular to it, resembling an actively segregated nucleoid mimicry. Thus, we will build cell-like systems that can maintain physicochemical homeostasis while undergoing the major physical transformation of self-division. Achieving these goals will be an essential step towards designing genuinely life-like synthetic cells and an unrivaled tool to scrutinize our fundamental understanding of the basic mechanisms of life. These insights on an essential cellular machine will open novel horizons to translate them into resources to curb bacterial proliferation. At the same time, the developed minimal cell-like systems constitute a powerful platform for the future engineering of systems, devices, and materials with novel functionalities of biotechnological added value.

General aim: to design a vesicle division machinery operated by self-organizing proteins - in concert with a nucleoid mimicry and physicochemical elements of the intracellular complexity (as macromolecular crowding and phase separation) - to implement functional autonomous division of minimal cell-like containers.

Specific aims

O1: Engineering the division ring for improved force generation
O2: Establishing strategies to aid constriction force induction through membrane patterning
O3: Engineering minimal actively segregated nucleoid mimicries
O4: Spatial orchestration of modules for autonomous division

In O1-3 we will reveal the minimal force on the membrane that is needed to constrict the vesicles and the machinery that is needed to exert this force. In O4 we will develop a blueprint for the *in vitro* integration of energy producing and dissipating machinery under far from equilibrium conditions, which is not available at present.



DIVINOCELL roadmap. Assembly of the key components for autonomously dividing minimal cells. A spatiotemporally self-organizing division machinery will be encapsulated in vesicles and drive their self-division through a combination of contractile and expansion forces. An engineered division ring will produce the contractile forces. An actively segregated DNA mimicry will contribute to the lateral expansion forces.

1. SCIENTIFIC PROPOSAL: Hypothesis and background

DIVINOCELL will use the already known part of the cell division engine and the knowledge of their assembly to reconstitute, from the bottom-up, simplified machines able to mimic the essential functions of the natural engine in cell-like containers (*i.e.* in the absence of cells). Therefore, DIVINOCELL frames within one of the most formidable and exciting challenges of 21st-century science and technology: integrating individual molecular systems into functional modules towards building a minimal living cell from scratch to reveal the boundaries of life. Our laboratory was critical in the assembly of a national consortium on synthetic cell research (integrated into the CSIC network LifeHUB) and instrumental to the incorporation of the CSIC to the steering committee of the European Synthetic Cell Initiative (SynCellEU – www.syntheticcell.eu) together with the Max Planck Society, the Dutch consortia on synthetic cell research (involving around twenty research centers) and the French Alternative Energies and Atomic Energy Commission (CEA). This European network has joined forces to collaborate on synthetic cell research to understand the basic principles of life and its emergence from lifeless components and accelerate the translation of fundamental studies into technology. These efforts are in line with the CSIC strategic plan and challenges for the next decades (*i.e.*, Theme 2 - Origins, Co-Evolution, Diversity, and Synthesis of Life - of the White Paper CSIC Scientific Challenges 2030; the CSIC Life-Hub, and the PTI+ *SusPlast).*

Knowledge generated and tools derived from DIVICELL will contribute to the mechanistic understanding of the molecular interactions governing the operation of essential subcellular machines and decipher the fundamental principles of subcellular organization and how physicochemical elements of intracellular complexity (as macromolecular crowding and phase separation) modulate macromolecular organization and cell function. Overall, the DIVINOCELL research program uniquely aligns with the topics of the ERC scientific panels LS1 (*Molecules of life – mechanisms, structures, and functions*) and LS9 (*Biotechnology and systems bioengineering*). Besides, synthetic cell technology can contribute to a healthier and greener future by providing sustainable innovations inspired by nature. In particular, the knowledge and tools resulting from DIVINOCELL (*i.e.*, synthetic divisomes, minimal cell-like containers, crowding and biomolecular condensation driven by phase separation) will translate into resources to design novel antimicrobials to combat bacterial infections and to design synthetic self-assembling systems with technological added value.

1.1. Starting hypothesis

Living systems have the unique ability to self-organize, grow, replicate, and evolve through a continuous flow of energy and matter [1], a feature that has never been possible to recreate in man-made systems. One of the grand scientific challenges is to design and study such open systems that are 'out-of-equilibrium' under defined conditions and resolve the minimal mechanisms that allow a living cell to sustain itself, grow, and divide. The vast methodological advances in molecular biology, biophysics and biochemical reconstitution of the past decades with regard to the knowledge and manipulation of key cellular modules have made the **full bottom-up construction of a minimal living system** a tangible, yet enormous, goal for interdisciplinary research [2-9]. It is hypothesized that this goal can be achieved by dissecting key cellular functions and features into manageable subsystems or modules, which are synergistically combined step-by-step into larger and more complex entities.

In DIVINOCELL, we propose to develop **minimal cell-like systems capable of energy dissipation, physicochemical homeostasis and autonomous cell division** based on bacterial-sized membrane vesicles. We will assemble, from the bottom-up, synthetic vesicles harboring **active functional machineries to spatiotemporally self-organize and produce the forces for self-division** together with actively segregated DNA nucleoid mimicries that will support lateral expansion forces. Our system will allow us to dissect native cellular mechanisms to their key principles with unprecedented quantitative rigor. Eventually, the combined forces executed by the energy-dissipating machineries will result in an actively orchestrated self-division, a key hallmark of life.

For this ambitious goal, we will exploit our demonstrated expertise in the biochemical reconstitution of minimal cell division, and build on recent accomplishments in protein-based bottom-up synthetic biology [2, 5, 10, 11]. Additionally, we will implement exciting new insights obtained by the recognition of liquid-liquid phase separation (LLPS) as a fundamental generic regulator of physiological processes [12-15].

1.2. State-of-the-art and advances beyond

The past decades have supplied us with a wealth of knowledge to approach the challenge of building even the smallest and simplest living system from scratch, aided by cutting-edge technology and methodology from chemistry, computational sciences, molecular biology, and nanoengineering [3-5, 11, 16]. Origin-of-life research primarily attempts to establish the emergence of complex biomolecules from plausible prebiotic precursors [17, 18]. Protocell research, on the other hand, recapitulates physicochemical driving forces of life in vesicle systems of low chemical complexity for the ability to grow, divide and even process biological information [19, 20]. Inspired by advances in biochemical reconstitution and molecular biology, **bottom-up synthetic biology** instead aims to understand life as an assembly of active modules and machines with the remarkable ability to **establish and sustain spatiotemporal order far away from thermodynamic equilibrium,** characterized by the concept of **functional self-organization** [3, 21-24].

There have been exciting advances with regard to the functional reconstitution of active cellular elements in vesicle compartments [10, 25-27]. To approach division based on functionally active protein machinery, several groups worldwide have begun to explore the reconstitution of archetypal division systems from bacteria to eukaryotes and even archaea [2, 6, 7, 28, 29]. Of particular interest are the force-producing cytoskeletal filaments. Most of these energy-dissipating machineries in living organisms depend on contractile protein structures that catalyze membrane fission. In eukaryotes, the efforts have concentrated on actomyosin [30, 31], dynamin [32], and ESCRT-III [33] (reviewed in [2]), revealing that contractility in these systems mainly depends upon motor protein activity on their template filaments. However, a complete mechanistic understanding of even the most obvious contraction activity, as in actomyosin rings [34], is still lacking [31].

In bacteria, FtsZ, which constitutes the core of the bacterial Z ring [35-37] has received much attention. In the absence of comparable filament-motor systems in prokaryotes, the mode of contractility in filament structures such as FtsZ is supposed to be conceptually more straightforward. They consist of so-called "cytomotive filaments" [38, 39] in which conformational changes by nucleotide hydrolysis in the filaments themselves may induce mechanical forces. The Z ring is supposed to execute scission into two equally-sized daughter cells by combining force-inducing processes that are not yet fully elucidated. Although the most significant contribution *in vivo* may result from cell wall-synthesizing enzymes to accomplish inward growth of the stiff peptidoglycan-based cell wall [40], competent division of cells devoid of a wall points to mechanisms that directly act on the cell membrane [41-43]. These are of paramount interest to our vesicle systems. The primary protein of the Z ring, FtsZ, a filament-forming tubulin homolog that displays rich assembly-disassembly dynamics upon GTP consumption, can contribute to force generation [44, 45]. In this regard, we have demonstrated that treadmilling dynamics of FtsZ filaments without reconstituted Z rings lead to the transformation of bacterial-sized membrane tubes [42].

Several groups (including ours) approached the goal of minimal protein-based cell division by **re-constituting essential elements of the divisome from** *Escherichia coli* **in the test tube** (reviewed in [7, 28, 29, 46]; see also ***Section 3.5*** and references therein), which is a remarkably well-adapted system for a minimal-cell design. It consists of well-characterized protein systems for contractile ring assembly and positioning in which FtsZ and the negative (Min and nucleoid occlusion systems) and positive (membrane proto-ring elements, FtsZ associated proteins, and the Ter-linkage) regulators of ring stability work together as an integrated system of molecular interactions, including those involving the lipid membrane and the bacterial chromosome [35, 47] (**FIG. 1**). Our laboratory has significantly contributed to obtaining a complete biochemical description of these interactions and to defining how physicochemical elements of intracellular complexity (as macromolecular crowding, surface interactions, and biomolecular condensation mediated by phase separation) modulate these interactions and the spatiotemporal organization underlying the operation of minimal divisome

machines (see ***Section 3.5*** and references therein). This knowledge is crucial to achieving the goals of DIVINOCELL.

Very recently, and as one of the starting points for this proposal, the Schwille group (P. Schwille is a member of the work team) accomplished the co-reconstitution of a complete set of five division-related proteins from *E. coli* in giant unilamellar vesicles, both with purified components and in a system based on cell-free protein expression [10]. The Min proteins pole-to-pole oscillations aid the assembly of FtsZ into large ring structures and position them to the equatorial region of a membrane vesicle. Interestingly, excluded volume effects due to natural crowding were crucial to achieving the Min-FtsZ self-organization coupling inside the vesicles. This is in line with our previous findings regarding the impact of crowding on FtsZ bundling and its enhancing-effect on the formation of FtsZ waves driven by Min oscillations in minimal membranes [48]. However, from the study of [10] it also became apparent that these vesicles will likely not support a sufficient radial constriction by a minimal FtsZ ring only and that **other forces will have to be in place for full division.**

1.3. Novelty and hypothesis

We hypothesize that these additional forces should come from a combination of **factors to improve a) the contractile strength and activity of the ring** and **b) the perpendicular expansion of the membrane compartment.**

a) We will improve force generation by the action of **regulators of ring formation in the cell**, either positive (ZapD, elements of the Ter complex) or negative (accelerating FtsZ disassembly, such as SlmA), and will aid constriction force induction through membrane transformation driven by controlled mid-cell accumulation of divisome proteins (as ZipA) or protein LLPS at the membrane.

b) Genome segregation mediated by the Min oscillatory behavior coupled to physicochemical forces (linked to macromolecular crowding and liquid-liquid phase separation) will aid the expansion forces perpendicular to the FtsZ ring. As genome segregation is inherently a hugely complex process, we propose to scrutinize this hypothesis by designing **actively segregated minimal nucleoid mimicries** based on engineering bacterial plasmids or DNA-based hydrogels.

Our proposal builds on our internationally recognized strength in bottom-up synthetic biology of cell division, as well as biochemical reconstitution technologies and quantitative microscopy-based analysis methods developed and mastered by our group. For our minimal cell design, we further benefit from recent technological advancements in microfluidics and microsystems engineering [28, 51, 52]. We have also contributed new important insights in physical and cell biology, such as the recognition of liquid-liquid phase separation as a fundamental generic regulator of essential physiological processes including those occurring in bacteria [53-56]. Building cell-like systems that are able to maintain physicochemical homeostasis while undergoing the major physical transformation of self-division will be a **huge step towards the design of synthetic cells**, and an unrivalled tool to scrutinize our fundamental concepts of the phenomenon of life. The impact of DIVINOCELL goes beyond fundamental mechanistic insight in cellular homeostasis and the functional origin of cell division. Our synthetic vesicles with essential cell-like functionality will **represent a new generation of biology-based technology**.

The highly ambitious research program of DIVINOCELL requires a solid conceptual foundation and experimental proficiency in the general scientific methodology of **bottom-up synthetic biology**, as well as the respective biochemical and biophysical expertise in the design and analysis of minimal cell division and physicochemical homeostasis linked to macromolecular crowding and phase separation. We have pioneered the biochemical description of the multiple interactions between FtsZ and the elements regulating the stability of the division ring (including those involving protein-DNA-membrane interactions). This knowledge has led to the **functional reconstitution and re-engineering of protein networks involved in bacterial cell division.** Besides, we have significantly contributed to deciphering the mechanisms underlying intracellular homeostasis driven by macromolecular crowding and phase separation concerning cell division events. These

achievements and expertise are at the heart of this proposal to achieve autonomous division in minimal cell-like systems.

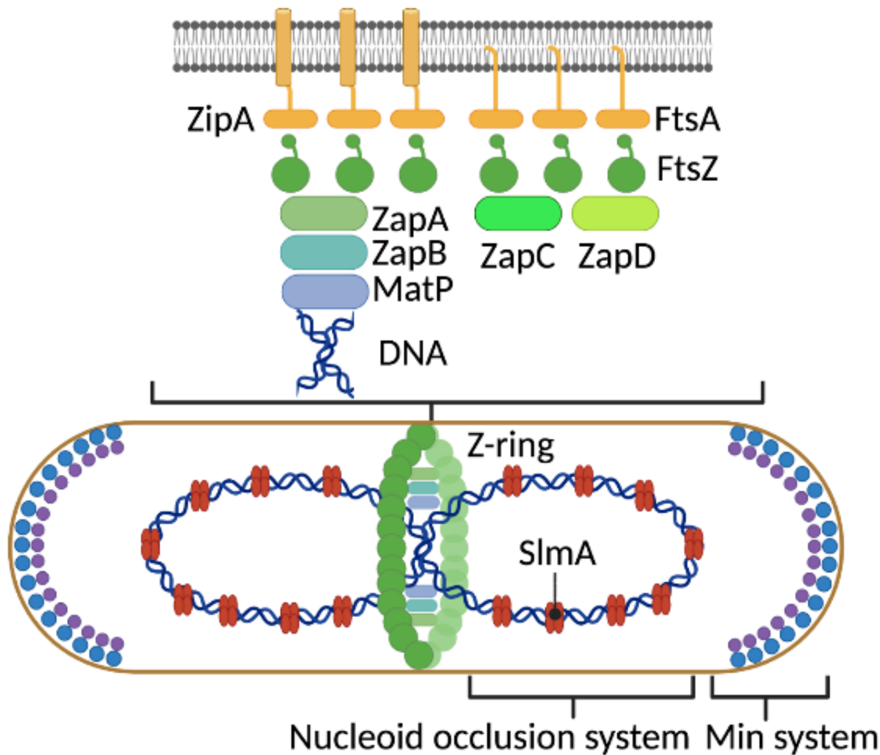


FIG. 1: Organization of the Z-ring and its regulation mechanisms before cell division. In most bacteria, FtsZ is the major component of the divisome (molecular machinery effecting cytokinesis). FtsZ interacts with additional proteins that contribute to its function forming a dynamic ring that is essential to constrict the membrane. In E. coli, the midcell is the zone active in assembling the ring in which FtsZ, FtsA and ZipA form the initial proto-ring. ZipA and FtsA serve to anchor the ring to the membrane, and together with a set of Zap proteins, they stabilize it. MinCDE and the nucleoid-associated SlmA protein prevent the polymerization of FtsZ at sites other than midcell. MatP (forming the Ter-linkage together with ZapA and ZapB) promotes the assembly of the Z ring nearby the replication terminus of the chromosome. Above, scheme magnifying how FtsZ is anchored to the membrane through ZipA and FtsA, and is connected to the Ter-linkage [35, 36, 49, 50].

2. OBJECTIVES

We aim to design a vesicle division machinery operated by self-organizing proteins - in concert with a nucleoid mimicry and physicochemical elements of the intracellular complexity (as macromolecular crowding and phase separation) - to implement functional autonomous division of minimal cell-like containers. The intended main outcomes are illustrated in **FIG. 2**.

Specific aims

- O1: Engineering the division ring for improved force generation
- O2: Establishing strategies to aid constriction force induction through membrane patterning
- O3: Engineering minimal actively segregated nucleoid mimicries
- O4: Spatial orchestration of modules for autonomous division

In O1-3 we will reveal the minimal force on the membrane that is needed to constrict the vesicles and the machinery that is needed to exert this force. In O4 we will develop a blueprint for the *in vitro* integration of energy producing and dissipating machinery under far from equilibrium conditions, which is not available at present.

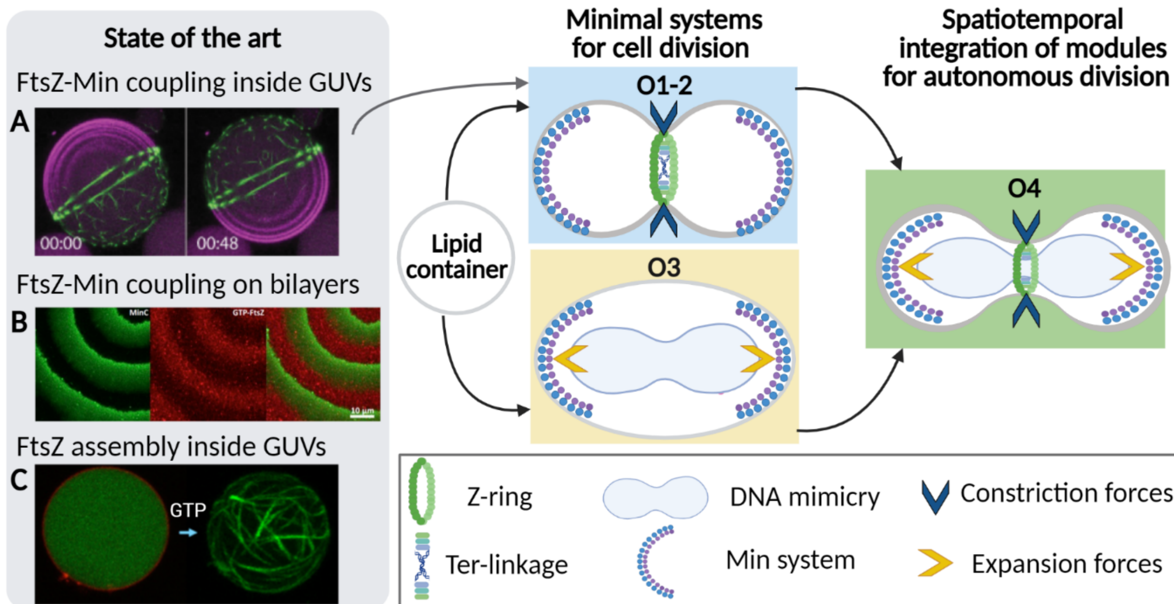


FIG. 2: Schematic of the DIVINOCELL roadmap. Assembly of the key components for autonomously dividing minimal cells. A spatiotemporally self-organizing division machinery will be encapsulated in vesicles and drive their self-division through a combination of contractile and expansion forces. An engineered division ring will produce the contractile forces. An actively segregated DNA mimicry will contribute to the lateral expansion forces. Images on the left taken from [10] (A), [48] (B), [57] (C).

3. PREVIOUS RESULTS OF THE TEAM

Our group has a long trajectory in the analysis of the interactions of the elements of the bacterial division machinery in solution and by reconstitution in membrane systems, and in the determination of the impact of intracellular crowding and phase separation on these interactions. We have purified the central bacterial division protein FtsZ from *E. coli* and the other proto-ring proteins (ZipA and FtsA), the regulators of Z-ring stability (SlmA, MatP, ZapA, ZapB, MinCDE complex), some mutants, fluorescent chimeras, division proteins from other organisms (*i.e.*, *S. pneumoniae*) and bacteriophage λ Kil peptide, and we have covalently labeled them with a variety of fluorophores. We have developed biophysical assays to decipher the interactions among these proteins, with DNA and with membranes and implemented technologies for their reconstitution in minimal membrane systems and for their encapsulation by microfluidic technologies that allow the reconstitution of proteins in cell-like containers and their subsequent analysis using advanced microscopy and fluorescence microspectroscopy methods. These materials, skills, and experience are essential to address the proposed experimental challenges.

The main achievements of our laboratory concerning the current proposal are the following (**FIG. 3**):

Biochemical and biophysical analysis of FtsZ associations and assembly. Control by nucleotides and magnesium of FtsZ self-association and assembly in solution evidenced by sedimentation velocity [114] and orthogonal approaches based on static light scattering and hydrodynamic techniques [115, 116]. Description of the interaction mechanisms of FtsZ and negative regulators of Z-ring stability (MinC, SlmA, and bacteriophage λ Kil [74, 117, 118]). Designing of plasmonic biosensing assays to screen for inhibitors of bacterial division proto-ring assembly [119, 120]. Crosslinking by ZapD drives the assembly of short, discontinuous FtsZ filaments into ring-like structures in solution [70].

Biochemical reactions in cytomimetic media: macromolecular crowding. Development of analytical ultracentrifugation methods to study protein behavior and interactions in highly crowded solutions reproducing natural environments [63-65, 121]. Development of a fluorescence anisotropy procedure to monitor FtsZ polymerization in solution and in crowding conditions [66]. Development of time-resolved fluorescence spectroscopy and microspectroscopy (FCS) approaches to study protein reactivity in crowded solutions [67, 122]. First observation of the impact of physiological crowding on the self-association [64] and assembly [68] of the essential bacterial division FtsZ protein, as revealed by reconstitution biochemistry. Further exploration of the effect of homogeneous mixtures of crowders – beyond volume exclusion – on FtsZ assembly [69]

FtsZ and macromolecular phase separation. First observation that FtsZ, together with nucleoid-associated SlmA protein, forms phase-separated condensates in crowded cell-like media [53]. Identification and characterization of homotypic FtsZ biomolecular condensates [54]. First demonstration that glutamate and lipid bilayers enhance FtsZ biomolecular condensation [55]. Demonstration that FtsZ biomolecular condensates can act as hubs that partition division factors, regulating their interconversion with polymers in response to crowding [56].

Reconstitution of minimal divisomes in membrane systems and cell-like compartments. Dynamic interactions between the bacterial division FtsZ and ZipA proteins evidenced in phospholipid bilayer nanodiscs [58, 123]. First encapsulation of FtsZ in microfluidics microdroplets stabilized by a lipid boundary resembling the *E. coli* inner membrane [105] and dynamic distribution of FtsZ in membraneless microenvironments [59]. Constriction forces partially reproduced by defined bacterial division elements (FtsZ, ZipA) when assembled in permeable cell-like vesicles [57]. Oscillating Min proteins can drive FtsZ waves in ZipA-containing bilayers [48]. Defining the role of protein surface density on the mechanisms of interaction between FtsZ and ZipA reconstituted in lipid-coated microbeads [60] and bilayers [61]. Demonstration that the formation of chiral vortices of FtsZ polymers is an intrinsic property of FtsZ [44]. The first description of dissipative self-assembly of FtsZ in coacervate-protocells [124]. Competition between DNA and lipid membranes for binding with MatP protein from the chromosomal Ter-linkage [73]. First description of the nucleoid occlusion regulator SlmA interacting with lipid membrane as part of its functional interaction network [104]. Encapsulation of a compartmentalized cytoplasm mimic within a lipid membrane produced by microfluidics [62].

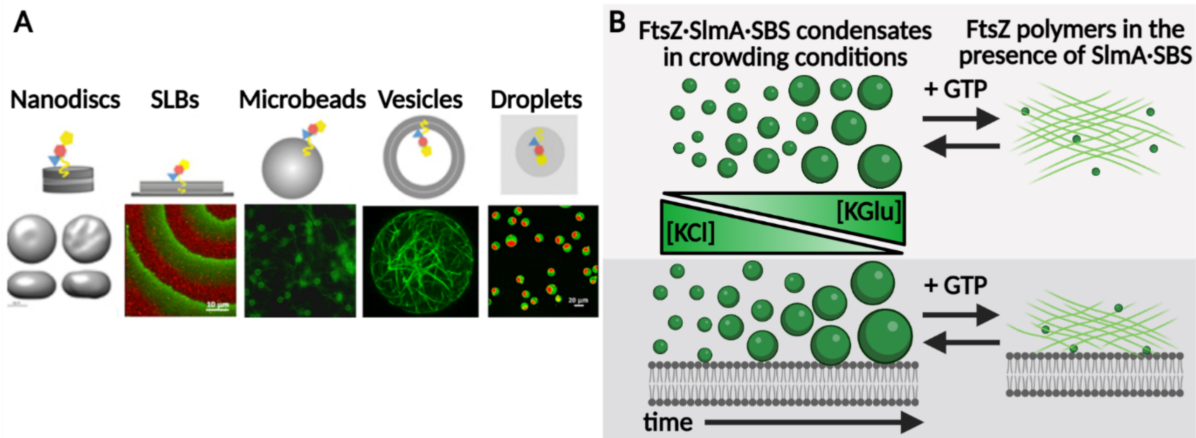


FIG. 3: (A) Proto-ring reconstructions in a variety of minimal membrane systems [48, 57, 62, 123]. (B) Scheme of phase-separated FtsZ·SlmA condensates, which are dynamic and disband upon GTP-dependent reversible formation of FtsZ fibers, and accumulate at the surface of supported bilayers [55].

Human, material and equipment resources available for the execution of the project

Our group is integrated by **four staff scientists** (G. Rivas, PI1; S. Zorrilla, PI2; C. Alfonso; M. Jiménez), holding complementary expertise in biochemical reconstitution of molecular machines, orthogonal biophysical methods including fluorescence and technological approaches such as microfluidics. We also count on two **PhD students** (G. Paccione and I. Barros), with a degree in Biochemistry. A **postdoctoral technologist** (M. Sobrinos) experienced in minimal membrane systems reconstitution approaches and biophysical methods will be also involved in the project. See ***Section 3.5*** for a description of our experimental / technological skills and long-term achievements related to the proposal. **We will also appoint one Ph.D. student, one postdoc and one technical assistant**. The Ph.D. student and PD will have a background in biochemistry, biophysics and/or biotechnology. The TA will carry out routine cell cultivation, protein purification, and quality control to support the research program.

Key collaborator for this proposal is **Petra Schwille** (director, MPI-Biochemistry, Martinsried), member of the work team. Given the challenges of the final goal of the research program (the autonomous division of a proto-cellular compartment), we will carry out the proposed project in close association with the Schwille group, our long-term collaborator in synthetic biology of cell division, primarily involving two of her junior associates who were former Master students of our lab (Adrián Merino and Maria Reverte). They will provide front-line microfabrication and microspectroscopy tools and optimized engineered variants of the Min protein system to assist in the controlled localization of divisome elements at certain membrane regions. Besides, the Schwille group will carry out the following parallel experimental efforts in close coordination with our lab (*see contingency plan – **Section 3.4***): 1) The design and implementation of cleavage ring constriction strategies using proteins synthesized by cell-free systems (while we will generally use biochemically purified proteins). 2) The reconstitution of minimal segregation machinery, based on the natural segregation system formed by the ParMRC proteins, and a synthetic nucleoid based on programmable DNA droplets.

Other collaborators for specific aspects of the proposal are the following: *Allen Minton (NIH, Bethesda, USA)*: physical chemistry of macromolecular interactions: impact of crowding and phase separation. *William Margolin (McGovern Medical School, UTHealth-Houston*): molecular microbiology of bacterial division. *Bert Poolman (Univ. Groningen*): physicochemical homeostasis (O4). *James Pelletier (CNB-CSIC, Madrid)*: quantitative biology of genomically minimal cells (O2.3). *Wilhelm Huck (Univ. Radboud, Nijmegen, NL)*: front-line vesicle microengineering. *Mario Mencía (CBMSO-CSIC/UAM, Madrid)*: design of plasmid based nucleoid mimicries (O3.1). *Julian Thiele (Leibniz Center, Dresden)*: design of chromosome mimicries based on programmable DNA hydrogels (O3.2). *Dolores Pérez-Sala* (CIB Margarita Salas-CSIC, Madrid): posttranslational modifications of proteins (O2.2). *Valentina Straniero & Ermanno Valoti* (Univ. Milan): design and synthesis of

benzamide derivatives targeting division proteins (O1, O4).

Available equipment. ***At CIB Margarita Salas:*** Our laboratory is well equipped for the production of recombinant proteins, for their biochemical characterization and fluorescent labelling and for the preparation of minimal membrane systems such as lipid-coated microbeads, nanodiscs, and the production by microfluidics of microdroplets and vesicles. We are experienced users of the 1) Molecular Interactions Facility with instrumentation for advanced analytical ultracentrifugation, light scattering (DLS, SEC-MALLS), optical biosensing and fluorescence (anisotropy and microspectroscopy - FCS, FCCS, time-resolved fluorescence) and 2) Confocal and Multidimensional Microscopy Facility. Other Facilities relevant for this project: Electron Microscopy, Protein Production and Chemistry, DNA sequencing, mass spectrometry, sterilization and culture media preparation and labware washing, technical support. Microplate readers for UV-visible absorption and fluorescence spectroscopies and equipment for protein purification are also available at the center. ***At extramural facilities or at collaborator labs:*** TIRFM imaging (*CNB-CSIC*, Madrid); quantitative imaging, single-molecule fluorescence spectroscopy, fluctuation and super-resolution microscopies complementary to those at CIB (*Euro-Bioimaging Facility, CBS-CNRS, INSERM, Montpellier*); nano/microfluidics and microfabrication platforms (*W. Huck lab, Radboud Univ. Nijmegen, NL*); advanced quantitative bacterial physiology tools (*S. Jun, Univ. California, San Diego*); time-resolved fluorescence, atomic force microscopy and quartz microbalance (*M. Vélez, ICP-CSIC, Madrid*); TIRFM-based binding assays (*M Loose, IST-Vienna*); rheometer, microfluidics-based vesicle production (*I López-Montero, UCM-Madrid*).

4. EXPECTED SCIENTIFIC IMPACT OF THE PROJECT

Knowledge generated and tools derived from DIVINOCELL will contribute to the mechanistic understanding of the molecular interactions governing the operation of essential subcellular machines and decipher the fundamental principles of subcellular organization and how physicochemical elements of intracellular complexity (as macromolecular crowding and phase separation) modulate macromolecular organization and cell function. Although the ultimate goal and large parts of DIVINOCELL are deeply rooted in fundamental, curiosity-driven research, the synthetic vesicles with essential cell-like functionality developed will represent a new generation of biology-based technology. Therefore, this work falls within the Technology Readiness Levels 1 to 3 as defined in the strategic plans of Horizon Europe.

Minimal cell-like systems capable of autonomous, energy-driven division constitute a powerful platform for engineering systems and materials with new functionality and precise controllability. Synthetic cell technology can contribute to a healthier and greener future by providing sustainable innovations inspired by nature. They will offer new opportunities for designing (bio)synthesis pathways, drug delivery, smart sensing-reporting systems, tissue formation, and even applications of symbiotic relationships with living cells. In particular, we foresee outcomes of DIVINOCELL (*i.e.*, synthetic divisomes, minimal cell-like containers, crowding, and biomolecular condensation driven by phase separation) will translate into resources to design novel antimicrobials to combat bacterial infections and to build synthetic self-assembling systems with technological added value. These developments align with Horizon Europe's objectives to *combat antimicrobial resistance and with the European green deal*.

The complex interdisciplinary setting of this project, requiring a combination of cutting-edge methods from biophysics, biochemistry, and bio-nanotechnology, results in many potential spin-off projects with practical application possibilities. These developments will be application-prone with various degrees of dissemination in academic, clinical, and industrial contexts.

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