Assembly and Reconstruction of Cytotoxic Bacterial Protein Assemblies in Lipid Vesicles

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Abstract

Biochemistry is at the core of constructive bottom-up approaches in synthetic biology aiming to generate a minimal cell. Any sort of cellular organization depends on compartmentalization of macromolecular assemblies and machineries within lipid, or amphiphilic lipid-like, vesicles. Following the increasing interest in the use of liposomes as cytomimetic devices, here we discuss our achievements toward using liposomes, especially giant unilamellar vesicles, as containers to reconstitute the amyloidogenic aggregation of the cytotoxic prionoid (prion-like protein) RepA-WH1. We address relevant issues as the importance of lipid composition and the effect or cofactors on assembly, and the feasibility of achieving protein expression within vesicles.

Abbreviations

CL

cardiolipin

C

curcumin

GMP-CPP

guanosine-5′-[(α,β)-methyleno]triphosphate

EGCG

epigallocatechin-3-gallate

GTP

guanosine-5′-triphosphate

GUVs

giant unilamellar vesicles

LUVs

large unilamellar vesicles

PC

phosphatidylcholine

PG

phosphatidylglycerol

Q

quercetin

R

resveratrol

sZipA

ZipA soluble protein

YFP

yellow fluorescent protein

1 Introduction

Bacteria currently are considered by many as too simple to be useful as models for understanding the biology of human health and disease, yet they occupy a privileged experimental niche between the simpler supramolecular world and the more complex (eukaryotic) cellular universe. Bottom-up approaches in synthetic biology have found in bacteria ideal targets in the attempts of reconstituting minimal but functional macromolecular assemblies, aiming to generate synthetic life [1–3]. In natural cells, the compartmentalization provided by lipid membranes is a fundamental requirement to have both a robust transmission of genetic information and a sustained metabolism. Therefore, lipid vesicles are attracting much attention as cell-like compartments or “chassis” for bottom-up synthetic biology [1,4,5].

These minimal membrane systems can be used as scaffolds to build simplified bacterial protein assemblies able to mimic the basic functions of natural machinery outside the cell [6,7]. In the last few years, new technologies in imaging and protein reconstitution in membranes have put this goal within reach, especially for machineries in which the description of its components is known, and their biochemical properties and their interactions are well characterized [8].

This is the case of the bacterial division machinery (the divisome), in which the knowledge of the molecular events related to the initial stages of division complex assembly has facilitated/prompted bottom-up synthetic biology approaches to reconstruct minimal divisome subsets in the test tube (reviewed in [6,7]). These developments have allowed reconstituting assemblies of FtsZ, the main component of the divisome in most bacteria that functions forming a dynamic ring at midcell that initiates division, and to test their functional organization in lipid vesicles, either artificially produced [9–12] or reconstructed from natural membranes [13]. Fig. 1 illustrates one of these attempts, in which FtsZ was placed inside micron-size vesicles together with ZipA, one of the proteins that naturally tether it to the cytoplasmic membrane of *Escherichia coli*. The vesicles were made permeable to allow triggering FtsZ polymer formation by external addition of guanosine-5′-triphosphate (GTP), the nucleotide that supplies energy to this process in bacteria. These vesicles shrank upon FtsZ polymerization, mimicking to some extent membrane constriction forces in cells [9].

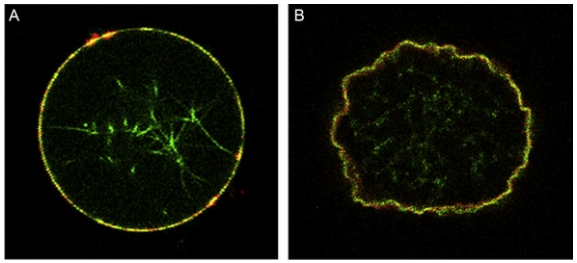

Fig. 1 Encapsulation and polymerization of FtsZ and vesicle shrinkage inside permeable vesicles. (A) Equatorial confocal image of a vesicle containing 2 μM sZipA-Alexa Fluor 647 and FtsZ-Alexa Fluor 488 after addition of GTP. (B) Equatorial confocal image of a vesicle-containing 10 μM sZipA-Alexa Fluor 647 with FtsZ-Alexa 488 after addition of GMP-CPP.

Further details in E.J. Cabré, A. Sánchez-Gorostiaga, P. Carrara, N. Ropero, M. Casanova, P. Palacios, P. Stano, M. Jiménez, G. Rivas, M. Vicente, Bacterial division proteins FtsZ and ZipA induce vesicle shrinkage and cell membrane invagination, J. Biol. Chem. 288 (2013) 26625–26634.

alt-text: Fig. 1

As a bottom-up synthetic biology challenge, we have recently explored if the experience accumulated on the reconstitution of functional bacterial divisomes in lipid vesicles could be shifted to reconstitute toxic protein complexes toward generating a minimal model of amyloid disease in bacteria, microorganisms that are naturally freed of such a kind of proteinopathy (reviewed in [14,15]). Amyloidogenic proteins can target biological membranes by inserting peptide segments through the lipid bilayer [16]. This is usually coupled to association of the protein into oligomeric ring pores, with a coupled conformational change from a coiled/α-helical conformation into amyloidogenic β-strands. Besides the loss of function commonly associated to protein aggregation, a gain-of-function phenotype is therefore another possible source of cytotoxicity. In particular, the formation of pores at membranes would leak the cytosolic (or the mitochondrial matrix) contents, either metabolites, cofactors, or small proteins, leading to impaired physiology (e.g., through the generation of reactive oxygen species) and, ultimately, to cell death. In a recent report [17], we described that the hyperamyloidogenic mutant variant A31V of the WH1 domain of the bacterial plasmid DNA replication initiator RepA [18] binds to lipid vesicles, promoting amyloidogenicity, and pore assembly. These findings have been independently confirmed through a global systems analysis of *E. coli* cells undergoing RepA-WH1 amyloidosis [19], illustrating the power of minimal, vesicle-based cytomimetic models to gain significant knowledge on the mechanism(s) for amyloid diseases. This chapter goes through the methodologies for RepA-WH1 binding and assembly into vesicles relevant to these discoveries. Table 1 summarizes the procedures for lipid vesicles production and the information provided by each model system.

Table 1 Summary of the Selected Methods for Vesicles Production Used in This Work

alt-text: Table 1

	Method	Advantages	Information Provided	References
LUVs	Prepared by extrusion techniques	▪ Simple ▪ Quantitative studies of protein–lipid interactions	▪ Binding and interaction of proteins with membranes	[17,20]
GUVs	Natural swelling in agarose films	▪ High yield of vesicles ▪ Suitable for natural membranes	▪ Membrane binding studies in the absence of oil	[17,21–23]
	Electroformation	▪ High yield of vesicles ▪ Suitable for lipids and natural membranes	▪ Membrane binding studies and incubation with antibodies avoiding the presence of oil	[17,21,24,25]
	Double emulsion method	▪ High encapsulation efficiency	▪ Encapsulation of fluorescent dyes for single vesicle leakage measurements ▪ Cell-free system encapsulation for protein expression in a biomimetic context	[9,17,21,26]

2 Protein–Lipid Interactions: Experimental Approaches Based on LUVs

Owing to the complexity of biological cellular membranes a wide range of model membranes have been developed trying to understand the contribution of lipids and protein–membrane interactions to many cellular processes in a controlled manner. The most common model membranes include lipid monolayers, lipid vesicles, and supported lipid bilayers [27,28]. Among lipid vesicles, large unilamellar vesicles (LUVs) have diameters between 100 nm and 1 μm and their size makes them suitable for the encapsulation of fluorescent dyes. A common preparation technique to obtain LUVs [20] consists in dissolving the phospholipids in an organic solvent and then to dry them down to form a film on the bottom of a glass vial. This film is hydrated using a suitable buffer containing the fluorescent dye to be encapsulated inside the LUVs. After hydration, the lipid sample is homogenized by freeze/thawing cycles; at the end of this step, multilamellar vesicles (MLVs) are formed. To obtain LUVs, MLVs have to pass through an extrusion step using two stacked polycarbonate filters of the desired pore size. Finally, the free dye can be separated by means of size exclusion chromatography; the process is illustrated in Fig. 2. The homogeneity of the LUVs suspension is usually determined by dynamic light scattering.

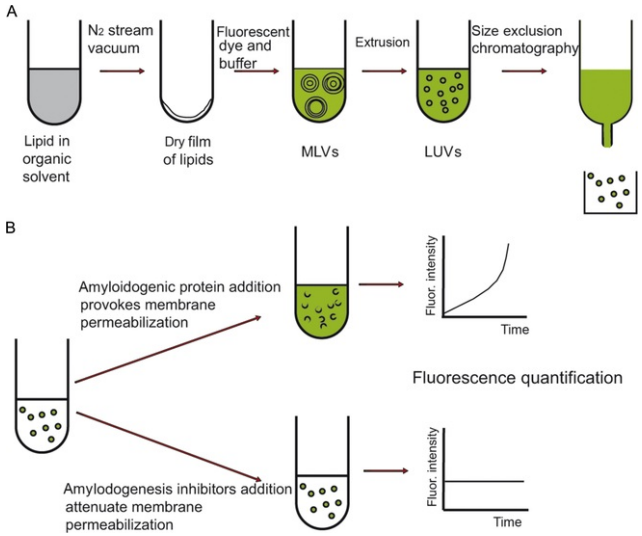

Fig. 2 (A) General strategy for LUVs production and dye encapsulation inside liposomes. (B) An outline of a procedure for monitoring protein (amyloid)-elicited dye release.

alt-text: Fig. 2

Because of their capacity to entrap fluorescent dyes, LUVs are a very popular model system for monitoring protein-induced membrane permeabilization through dye-leakage assays [28–30]. Fluorescent dyes, like fluorescein isothiocyanate, calcein, or carboxyfluorescein, when encapsulated in the interior of liposomes, have the ability to self-quench at high concentration due to intermolecular interactions, existing in the form of nonfluorescent complexes. Addition of a protein targeting and punching membranes causes dye dilution in the external medium and consequently an increase in the fluorescent intensity of the dye (Fig. 2B), an event that can be easily monitored in a fluorescence microplate reader [29].

2.1 Leakage Experiments to Study Protein–Lipid Interactions: The Case of Membrane Targeting by Amyloidogenic RepA-WH1(A31V)

This section is focused in the use of LUVs as model membranes to investigate the interaction of amyloidogenic proteins with lipids. As an example, we show the results obtained for the bacterial protein RepA-WH1(A31V), that is subject of research in our laboratory [17]. A large number of studies have identified the interaction of amyloids with the cellular surface by either a receptor or the lipid membrane, and found to be a possible mechanism of cytotoxicity [30,31]. LUVs are appropriate probes for getting insights into membrane targeting by amyloids. The most commonly utilized lipids for mimicking membranes are the uncharged, neutral phosphatidylcholine (PC), and phosphatidylethanolamine (PE), and the negatively charged phosphatidic acid, phosphatidylglycerol (PG), and phosphatidylserine (PS). *E. coli* inner membrane is composed of 60% neutral lipids (phosphatidylethanolamine), 33% of PG, and 7% of cardiolipin (CL) [32,33]. To vary the surface charge densities in LUVs, membranes containing a fraction of lipids with charged head groups like PG or PS are used, supplemented with zwitterionic lipids like PC.

The hyperamyloidogenic variant RepA-WH1(A31V) when fused to the fluorescent reporter mCherry, and expressed in *E. coli*, generates a synthetic amyloid proteinopathy that severely reduces bacterial proliferation and finally leads to bacterial death [19,34]. The amyloidogenicity of RepA-WH1 in *E. coli* cells is enhanced by populating a partially unfolded metastable state. This was achieved by fusing to its C-terminus a domain distinct to that found in wild-type RepA (WH2) [35]. We chose the monomeric protein mCherry, which allowed visualizing the prion-like protein by means of fluorescence microscopy [34,36,37]. In the resulting fusion protein, the mCherry tag has not a direct contribution to aggregation, because a fusion of mCherry to wild-type RepA-WH1 remained soluble and nontoxic in the cytoplasm [34,37].

Aiming to characterize RepA-WH1 amyloidogenesis in vitro, and inspired by the membrane targeting activity described for proteins involved in human amyloidosis [31], we have investigated the interaction of the bacterial RepA-WH1(A31V)–mCherry variant (the most cytotoxic in vivo), with suspensions of LUVs (≈ 100 nm) of various lipid compositions. In LUVs containing the self-quenching fluorescent dye calcein encapsulated, when calcein leaks from the interior of the vesicles results in an increase in fluorescence. We use as a control purified mCherry protein to compare the vesicle–permeabilization effects of both proteins [17,34–37].

For RepA-WH1(A31V)–mCherry, the lipid composition that leads to maximum fluorescence emission upon incubation with LUVs include mixtures of the zwitterionic phospholipid PC and acidic phospholipids (with negatively charged polar heads), such as PG or CL (Fig. 3A and B). LUVs made only of negatively charged phospholipids (PG) result in intrinsic destabilization of the vesicles, leading to some leakage induced even by the control protein mCherry. In fact, such 100% composition of negatively charged phospholipids does not reflect the situation present at cellular membranes. Time–course measurements on LUVs made of a 50% mixture of PG and PC, which is a more realistic composition for mimicking the *E. coli* internal membrane, showed that RepA-WH1(A31V)–mCherry induces the damage of the vesicles immediately after the addition of the protein. Leakage kinetics exhibits a logarithmic profile and the release of calcein is completed within minutes. However, at these conditions, mCherry barely exhibits any calcein releasing activity by itself [17] (Fig.

3A).

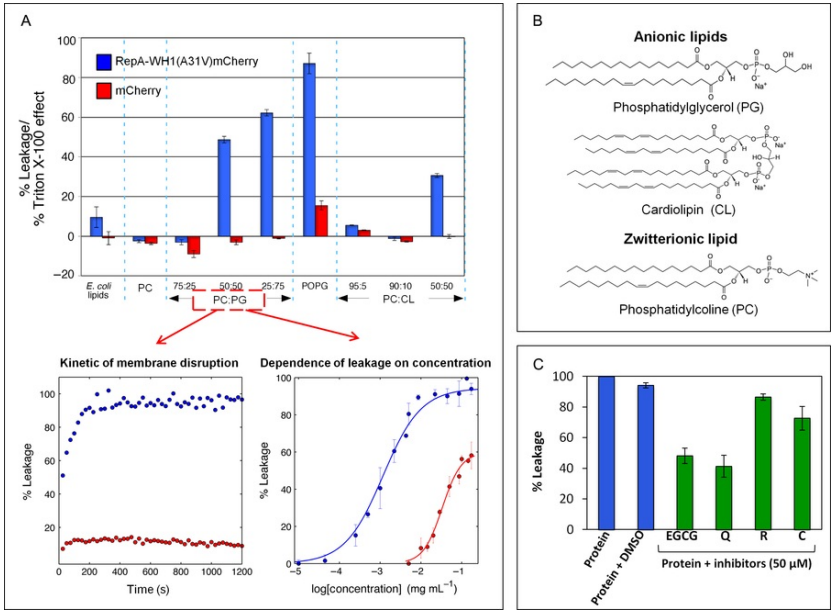

Fig. 3 LUVs as experimental systems to address membrane targeting by the amyloidogenic RepA-WH1 protein. (A). Calcein leakage from LUVs made of different lipidic compositions. RepA-WH1(A31V)–mCherry induces leakage of LUVs that contain negatively charged phospholipids (PG or CL). Kinetics of permeabilization can be followed upon protein addition. To compare the effect of different proteins on vesicle leakage, we determined membrane disruption in a range of protein concentrations in GUVs made of 50% PC:PG. (B) Chemical structure of some lipids found in biological membranes used to form LUVs. (C) Polyphenolic compounds inhibit the interaction of RepA-WH1(A31V)–mCherry with membranes.

Figures were adapted from C. Fernández, R. Núñez-Ramírez, M. Jiménez, G. Rivas, R. Giraldo, RepA-WH1, the agent of an amyloid proteinopathy in bacteria, builds oligomeric pores through lipid vesicles, Sci. Rep. 6 (2016) 23144.

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By systematically altering the vesicle lipid composition and solution conditions, it is possible to define the fundamental driving forces directing protein–lipids interaction. For the RepA-WH1, prionoid membrane permeabilization is mainly determined by the presence of negatively charged lipids in the bilayer at pH 6 (20 mM NaH_2PO_4, 0.1 M Na_2SO_4). These interactions appear to play a crucial role in the binding of RepA-WH1 to lipids. Previous work identified a highly electropositive surface in the RepA-WH1 dimer, contributed by eight arginine residues, as involved in the transiently binding to the allosteric dsDNA sequences in amyloidogenesis. In the whole RepA, the same residues sit over the phosphate backbone of DNA when the protein acts either as a replication initiator or as a transcriptional repressor. Therefore, it is likely that the arginine patch would be also involved in the electrostatic interaction of RepA-WH1 with the heads of the acidic phospholipids [35,38].

A disadvantage of this type of studies is the limited information that can be obtained about the permeabilization mechanism (e.g., ion channels/pores, membrane destabilization, or detergent-like mechanism). To get more insights about the mechanism of interaction of amyloidogenic proteins with membranes, including single-vesicle measurements, it is necessary to work with additional model systems, as we will show in Section 3.

2.2 Leakage Experiments for the Analysis of Amyloidogenesis Inhibitors

One of the most interesting advantages of in vitro assays based on LUVs is their suitability to test compounds that can interfere with the membrane activity of amyloidogenic proteins involved in diseases. The search for therapeutic agents that inhibit amyloid assembly is of great therapeutic interest, but poses an important challenge. In this context, the interaction with the membrane should be taken into account as one of the key determinants of toxicity and relevant target for drug discovery. In parallel with the development of synthetic compounds, research has also been carried out on natural-based inhibitors, in particular several polyphenols [39–41]. Polyphenols are a class of natural small molecules that are composed of one or more phenolic hydroxyl groups. Natural polyphenols are found in high concentrations in tea, red wine, different berries, and a wide variety of other natural products from plants.

For example, epigallocatechin-3-gallate (EGCG), which is extracted from green tea, was showed to inhibit the ability of α-synuclein oligomers to permeabilize vesicles and to have a protective activity on cells against amyloid disorders [42]. Other polyphenols with antiamyloidogenic activity include curcumin (C), resveratrol (R), or quercetin (Q).

We have tested the possible interference of several polyphenols on vesicle leakage in the presence of RepA-WH1(A31V)–mCherry: EGCG, Q, R, and C. All these compounds exhibited some inhibitory effect on calcein release from LUVs, ranging from 10% for R to 60% for Q, as illustrated in Fig. 3C. The observed reduction in membrane bilayer disruption when the compounds were preincubated with RepA-WH1 suggests that binding of the assembly modulators to the protein attenuate their membrane interactions.

The compounds that show the best inhibitory properties are Q and EGCG (Fig. 3C). In the interval between 5 and 60 μM, both Q and EGCG showed a linear response with concentration. Further control experiments confirmed that the polyphenols do not induce by themselves any detectable dye leakage in absence of protein [17].

3 Reconstitution of Cytotoxic Bacterial Protein Assemblies in Cell-Like Containers: Experimental Approaches Using Giant Vesicles

Since the development of the first reproducible procedure for the production of giant unilamellar vesicles (GUVs) [24], they have been extensively used as artificial membrane models suitable to investigate membrane-associated biological processes under defined experimental conditions in a confined space. They are composed by freestanding phospholipid bilayers and, due to their large size (from 5 to 100 μm of diameter), can be analyzed by means of optical microscopy. GUVs can be formed from different lipid mixtures, including bacterial membranes [13] and the available strategies for their manipulation allow their use as cell-sized biological reactors and minimal bacterial machineries [21].

3.1 Preparation of Giant Vesicles

In this section, we present a short summary of the procedures for preparing GUVs that have been used in the reconstitution studies related to the bacterial prion-like protein RepA-WH1 (Fig. 4).

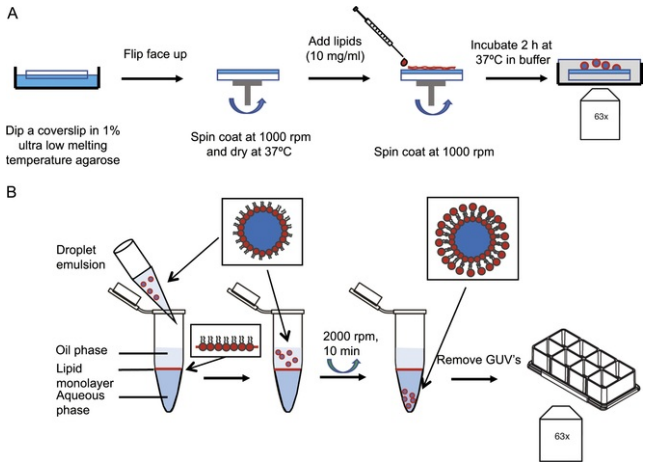

Fig. 4 Schematic representations of the main methods employed in this work for the formation of giant vesicles. (A) Natural swelling in agarose film method. Natural swelling in agarose and electroformation methods are based on the same principle to render vesicles from lipid layers deposited onto a solid surface. (B) Double emulsion method. Droplets are coated with the outer lipid monolayer by migration from the oil phase to the aqueous phase by low speed centrifugation.

alt-text: Fig. 4

3.1.1 Natural Swelling in Agarose Films and Electroformation

Natural swelling in agarose films and electroformation were used to characterize the interaction of RepA-WH1(A31V) with different types of lipids and the effect of the membrane lipid composition on its encapsulation in giant vesicles (see Section 3.2.1). These procedures reduce the contact with organic solvents to a minimum and are well adapted to reconstitute membrane-associated reactions involving transmembrane proteins and/or native biological membranes.

Natural swelling methods are based on the gentle hydration of a dry film of lipid molecules (or semidry native membranes) that spontaneously organizes to form vesicles. The method can be improved with the presence of a film of agarose. Hybrid films of agarose and lipids favor essential factors for vesicles formation (i.e., self-assembly of the lipids into lamellae, separation of lipid lamellae, and liposome fusion to form giant vesicles) [22,23].

The procedure starts by dipping previously ethanol-washed coverslips in an aqueous solution of 1% (w/v) ultralow melting temperature agarose and immediately spin-coating them at 1000 rpm for 30 s. The agarose is dried at 37°C generating a uniform film in one side of the coverslip.

A total volume of 10 μL of lipid at 10 mg/mL in chloroform is then spread onto the film by spin coating at 1000 rpm for 5 min. To remove residual solvent, the coverslip is dried under vacuum for 30 min. Finally, coverslips are placed into a Petri dish containing buffer solution where the hybrid film is faced upward. Liposomes were generated after incubating for at least 2 h at 37°C (Fig. 4A).

The electroformation method is based on the same principle just described above, but lipid layers are deposited into platinum electrodes or indium-tin-oxide (ITO)-treated coverslips. An alternating electric field with high frequency (500 Hz) results in the formation of giant vesicles even under physiological ionic conditions [25]. A few microliters of lipid mixture or native membrane are spread on each platinum electrode or ITO coverslip and residual solvent is evaporated for more than 30 min in a vacuum chamber. A homemade device~~s~~ based in a perfusion chamber is~~are~~ filled with buffer and giant vesicles are produced in 2 h at 37°C when the electric field is applied. A low frequency (10 Hz) during 10 min in the last step can be used for the total separation of the liposomes from the electrodes.

3.1.2 Double Emulsion Method

Double emulsion methods, based on the use of mineral oil, are suitable when coencapsulation of many compounds is required [26]. In this work, they have been used to assay membrane leakage by the bacterial amyloidogenic protein RepA-WH1(A31V) and to study the intravesicular expression of different variants of this cytotoxic prionoid (see Sections 3.2.1~~3.2.2~~ and 3.2.2~~3.2.3~~). The preparation of GUVs involves the assembly of two independently produced phospholipid monolayers mixing aqueous solutes and a mineral oil phase containing lipids. The most valuable advantage is the rather high encapsulation efficiency and the control on protein entrapment that it provides.

Stored lipids in chloroform were dried in a glass tube under a gentle stream of nitrogen gas to produce a lipid film and then kept under vacuum for at least 1 h to totally eliminate the organic solvent. Lipid films are dissolved in mineral oil (M5905, Sigma) at a total concentration of 0.5 mg/mL and incubated for 30 min in a water bath sonifier. After cooling down to room temperature, oil–lipid mixtures are stored at 4°C for up to a week. The outer monolayer is formed adding sequentially 500 µL of buffer solution plus glucose and 500 µL of the oil–lipid mixture in an Eppendorf tube. This provokes the assembly of a polar–hydrophobic interface. The inner monolayer is formed by a gently emulsification of 20 µL of protein solution in 500 µL of phospholipid-containing oil. This emulsion of drops is deposited on top of the previously filled Eppendorf tube at the oil–lipid mixture phase. Gently, centrifugation of the tubes (2000 rpm, 10 min) forces the droplets in the emulsion to pass through the interfaced monolayer, resulting in a bilayer surrounding the entrapped protein solution at the bottom. After removing the oil suspension from the top, vesicles are rinsed with the buffer to remove oil contaminations and nonencapsulated proteins (Fig. 4B) [9,26].

3.2 Assembly of a Cytotoxic Amyloidogenic Bacterial Protein Inside GUVs

3.2.1 Encapsulation of the Prion-Like Protein RepA-WH1: Effect of Different Lipid Composition and Membrane Leakage Assays

We have investigated important aspects of the process of amyloid aggregation using giant vesicles as an artificial membrane system. With this approach, we obtained additional information that the LUV suspension method cannot provide, such as single liposome resolution, essential to probe homogeneity in the population of vesicles, or information about the mechanism how the protein exerts damage to membranes [31,43].

With natural swelling in agarose films, we prepared GUVs in the presence of the amyloidogenic protein RepA-WH1(A31V)–mCherry and directly visualized the results with confocal fluorescence microscopy. Depending on the lipid composition, the protein remains in the interior of the vesicles (as soluble protein) or becomes visible on the surface of GUVs. When vesicles were prepared with purified bacterial internal membranes, or with phospholipids having the average composition characteristic of the *E. coli* inner membrane, 50% PG or CL, the protein appeared bound to lipids and aggregation at the surface of the vesicle became evident (Fig. 5A). However, RepA-WH1(A31V)–mCherry remained soluble if the lipids are exclusively neutral (PC), whereas the purified mCherry control stayed soluble in all the conditions tested [17].

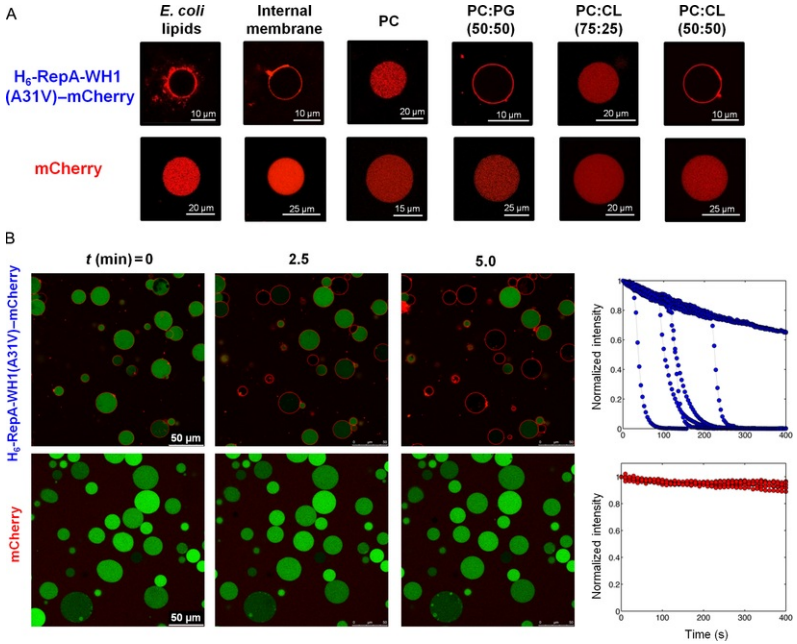

Fig. 5 (A) RepA-WH1(A31V)–mCherry interact with vesicles formed from hybrid films of agarose and lipids. Lipids were incubated at 37°C during GUV formation with proteins. (B) Leakage experiments on GUVs. Fluorescence emission decreases when calcein is released into the medium after RepA-WH1(A31V)–mCherry addition (*red*). GUVs were made of 50% PC:PG, filled with calcein, using the double emulsion method. The *plots* on the right show the time course of the change in the normalized fluorescence intensity of several GUVs.

Figure adapted from C. Fernández, R. Núñez-Ramírez, M. Jiménez, G. Rivas, R. Giraldo, RepA-WH1, the agent of an amyloid proteinopathy in bacteria, builds oligomeric pores through lipid vesicles, Sci. Rep. 6 (2016) 23144.

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Leakage from vesicles can also be directly visualized using GUVs. For these experiments, calcein is entrapped inside GUVs and visualized with fluorescence microscopy as a green fluorescence emission. For high efficiency encapsulation, we prepared 50% PC:PG-GUVs with the droplet transfer method. Once the vesicles were ready, they were washed several times to remove completely any remaining free calcein. The time course of leakage of calcein incorporated inside of GUVs was observed using time-elapsed fluorescent microscopy after the addition of RepA-WH1(A31V) fused to mCherry to the external medium as a reporter. Soon after protein addition, the binding of RepA-WH1(A31V)–mCherry to the lipid bilayer of GUVs (red fluorescence emission) was observed, and many vesicles started to leak calcein (green fluorescence) that was encapsulated inside (Fig. 5B). An important observation during the course of the experiment was that the shape of the giant vesicles does not seem to be altered, clearly pointing to the formation of pores as the possible mechanism of leakage, as it was later shown by 2D projection analysis of images acquired by electron microscopy [17].

It is worth noting that experiments with LUVs and GUVs led to comparable results, RepA-WH1(A31V)–mCherry caused damage to lipid membranes containing negatively charged phospholipids. Moreover, we obtained additional valuable information with GUVs concerning the mechanism of leakage.

3.2.2 Cell Free, Intravesicular Expression of RepA-WH1

An important step toward the better understanding of biological systems using GUVs is the successful encapsulation of an entire gene expression system to carry out the process of transcription and translation to produce proteins in a cell-like environment, i.e., comparable to that of a cell. Up to now, the synthesis of functional proteins within liposomes has been examined in several reports using cell-free systems [44–48].

We have combined the droplet transfer method, for the production of GUVs, with the use of the PURE system, to express RepA-WH1. The PURE system [49] is a commercially available reconstituted system that contains the minimal set of factors necessary for in vitro protein production: purified enzymes, ribosomes, and tRNAs (Bio-Labs PURExpress® In Vitro Protein Synthesis Kit Cat. E6800S). The gene of interest was cloned in a plasmid downstream to a T7 promoter and this plasmid was mixed together with the components of the coupled transcription and translation machineries. Plasmid and cell-free system were encapsulated inside the vesicles as indicated in Section 3.1.2 and the intravesicular synthesis of the protein proceeds during incubation at 37°C (Fig. 6).

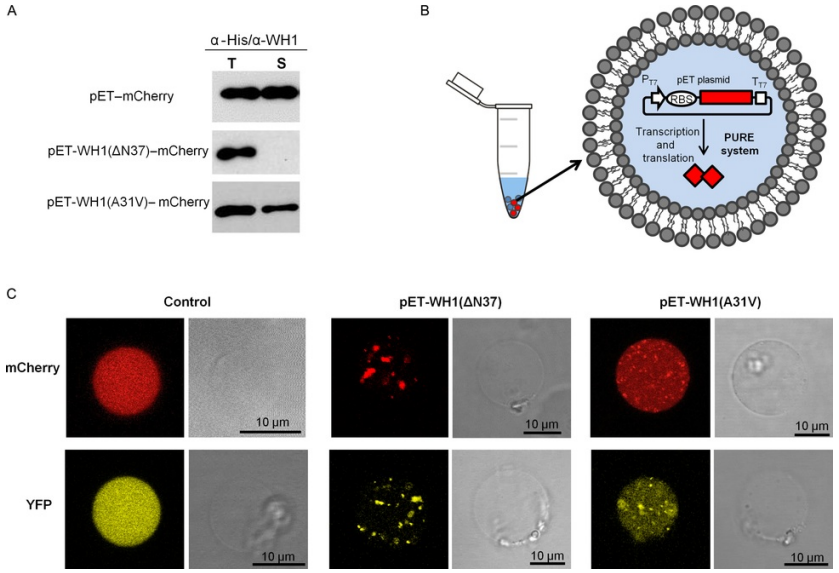

Fig. 6 Cell-free expression of RepA-WH1(A31V). (A) Cell-free expression carried out in the absence of lipids. Protein synthesis of mCherry, RepA-WH1(ΔN37)–mCherry, and RepA-WH1(A31V)–mCherry was performed at 37°C for 3 h in a solution containing the PURE system (from New England BioLabs). After the reaction, synthesis products were analyzed by SDS-PAGE and Western-blot with α-His tag monoclonal antibody (Sigma) for mCherry and α-WH1 antibody [50] for the expression of RepA-WH1(ΔN37)–mCherry and RepA-WH1(A31V)–mCherry. T represents the total product of synthesis and S is the soluble fraction of the protein obtained after centrifugation of the total product at 30,000 × *g* for 30 min. (B) Schematic representation of cell-free expression inside liposomes of the gene of interest cloned in a plasmid under T7 promoter. (C). Protein synthesis inside GUVs after incubation at 37°C for 3 h. GUVs were formed by the droplet transfer methods and plasmids (encoding RepA-WH1 fusions to mCherry and YFP) and the PURE system were simultaneously encapsulated inside the vesicles. GUVs were observed by confocal microscopy in an 8-well visualization chamber (Lab Tech).

alt-text: Fig. 6

Different mutations in the RepA-WH1 gene result in variants of the protein with distinct amyloidogenicity when fused to fluorescent proteins (mCherry or YFP) [37]. Trying to reconstruct the behavior of the different proteins, as previously observed in bacteria, we encapsulated inside GUVs both the plasmids expressing the distinct variants of the bacterial prionoid and the cell-free system. RepA-WH1(ΔN37), a mutant variant that in bacteria aggregates as conventional inclusion bodies, formed big aggregates inside GUVs made of POPC, while the hyperamyloidogenic variant, RepA-WH1(A31V) formed smaller aggregates. As controls, neither individual mCherry nor YFP proteins aggregate when expressed in GUVs, but they remained soluble in the interior of the vesicles according to the diffuse fluorescence observed at the microscope (Fig. 6C).

The preliminary studies presented in this section open the possibility to investigate the effect of different factors involved in the aggregation of amyloidogenic proteins in a biomimetic context. In particular, single vesicle studies will address macromolecular interactions occurring before newly synthesized, metastable protein folding intermediates acquire their native conformation, which often lead to the assembly of oligomers on-pathway to the amyloid state.

4 Concluding Remarks

In this work, we have summarized the state-of-the-art toward engineering a synthetic device to test amyloid toxicity, in the context of cytomimetic membrane containers. Work carried out in different laboratories on amyloidogenic proteins involved in human disease suggests that the aggregation of such proteins on membranes and the formation of pores must be a general rule rather than an exception [16,51–54]. Developments along these lines will likely come from the use of more sophisticated and controllable lipid containers, as those based on microfluidic devices [12,55,56] in which prion-like bacterial protein complexes can be assembled and their function tested. Another advantage offered by the use of these synthetic reconstructions is that they can be devised to mimic near to natural crowded environments as the bacterial cytoplasm. Intracellular crowding causes volume exclusion effects, among others, which can significantly alter the extent and rate of protein assembly reactions, including those involved in amyloid formation [57,58]. This cell-free synthetic approach will help us to support conclusions already derived from cellular and molecular analysis and, therefore, to complete our understanding of amyloid formation in bacterial cells, which may open new pharmacological applications.

Acknowledgments

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