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Abstract

One of the open questions in origin of life is about the formation of primitive cells from separated molecular components. In recent years, this subject has been approached in the "Minimal Cell" project, namely the laboratory study on cell-like compartments with the minimal and sufficient number of components that may allow cellular life.

The theoretical framework of this research is the theory of *autopoiesis*, that elegantly defines what minimal life is in terms of self-bounded biochemical network capable of reconstructing itself (boundary included) thanks to its own reactions.

Current conceptual and experimental studies are focused on the study of cell-models created from lipid compartments, i.e., lipid vesicles (or liposomes). Until now, great attention has been devoted to consider the emergence of cellular life as an event that is bases on individual compartments, e.g., a single cell within a population that coentrap simple solutes that later internally develop a minimal metabolism, or complex solutes that are already part of an external metabolism. This view can be however not realistic, due to the low probability of such events. An alternative view explicitly considers the interaction between compartments as a way of stepwise increase of metabolic molecular complexity thanks to fusion or solute exchange processes.

The aim of this work is to put forward this new view, developing experimental strategies for its study and possibly revealing key aspects of this novel approach. No previous experimental reports on the subject can be found in the literature.
This can be done by achieving two main goals. Firstly, define and develop cell-model systems, and then propose a novel view about the possible important steps in primitive pre-cellular evolution, from the viewpoint of cell communities.

Giant vesicles (GVs) are used as cell model, because of their large size (1–100 um) which allows direct observation by light and fluorescent microscopy.

GVs have been produced with emulsion inversion method. This was selected because of its versatility and for the high solute entrapment that characterize it. However, this method was just discovered when this PhD work started and essentially no standard protocol was available. We have therefore, as a first step, outlined an experimental protocol that allows the highly reproducible production of GVs at high yield (10000 GVs/ul). It was possible to obtain GVs with an internal environment different from that outside, and entrap a wide range of water-soluble solutes as calcein (a small soluble molecule), FITC and RITC-dextran (sugars), allophycocyanin (APC), phycoerythrin (PE) (proteins) in their aqueous core. Preliminary results have extended this method to the entrapment of nucleic acids and enzymes.

In order to create a model of primitive colonies we exploited the electrostatic attraction, a very basic and long-range physical force that had to be present also in ancient time. As model cationic peptide we employed poly(arginine) (PLA) to trigger the aggregation of negatively charged GVs (POPC:oleate 1:1). Immediately after addition of PLA, GVs move towards each other, stick together and form GVs colonies of various size.

The process of the formation of colonies has been characterized by changing the variables affecting its occurrence. The first step was to test the threshold concentration of PLA. The second step was to verify whether the formation of colonies depended on the number of GVs initially present in the slide well. Thanks to these experiments we are able to reproduce the process of vesicle aggregation into colonies in a reproducible and controlled way.

Regardless of the amount of PLA added or the number of GVs initially present in the slide well, it was always possible to detect 1-5% GVs that derive, without any doubts, from the fusion of two or more GVs. This first important result confirms that the colony formation brings about an increase of complexity, giving an advantage to the colony with respect to individual GVs. Additional advantages of the GVs colonies with respect to individual (free) GVs lies in their resistance to flow, and against osmotic stress. Moreover, GVs colonies can grow by incorporating new GVs.

In conclusion, this work has opened a new vision with regard to phenomena at the base of the origin of life. It is the first time that from a vision of a single compartment it is passed to that of a community. A colony of individuals that work together is able to have selective advantages in respect to the individual one and thus evolve and achieve a greater level of complexity. We think that this model of primordial cell community is able to simulate a more likely the reality of cellular life.

Riassunto

Una delle questioni aperte nell'origine della vita riguarda la formazione di cellule primitive a partire dai componenti molecolari separati. Negli ultimi anni, questo tema è stato affrontato nel progetto "Cellula Minima", che prevede lo studio sperimentale di compartimenti cellulari, contenenti il numero minimo e sufficiente di componenti molecolari tali che possano consentire la vita cellulare.

Il quadro teorico di questa ricerca risiede nella teoria dell'autopoiesi, che definisce elegantemente ciò che è la vita minima in termini di network biochimici auto-organizzati e auto-confinati, in grado di ricostruire se stessi (compreso il proprio "confine" con l'ambiente) grazie alle loro reazioni.

Gli attuali studi, sia dal punto di vista concettuale che sperimentale, sono concentrati sullo studio di modelli cellulari creati dai compartimenti lipidici (vescicole lipidiche (o liposomi). Fino ad oggi, l'attenzione è stata dedicata all'emergere della vita cellulare come un evento che si basa su singoli compartimenti, per esempio, una singola cellula, all'interno di una popolazione, che cointrappoli semplici soluti che poi sviluppano, al suo interno, un metabolismo minimo, o complessi soluti che sono già parte di un metabolismo sviluppatosi all'esterno. Questo punto di vista può essere, tuttavia, non realistico, a causa della bassa probabilità di tali eventi di intrappolamento. Un punto di vista alternativo considera esplicitamente l'interazione tra compartimenti come un modo per aumento graduale della complessità molecolare metabolica, grazie a processi di scambio di fusione o di soluto.

Lo scopo di questo lavoro è di presentare questa nuova visione, e quindi di sviluppare strategie sperimentali per il suo studio, rivelando aspetti chiave di questo nuovo approccio. In letteratura non si trovano approcci sperimentali simili.

Questo goal può essere raggiunto mediante la realizzazione di due obiettivi principali. In primo luogo, definire e sviluppare sistemi cellulari modello, e quindi proporre una nuova visione sui possibili importanti passaggi nello scenario della evoluzione primitiva pre-cellulare. Questo deve essere fatto tenendo presente il nuovo punto

di vista delle comunità cellulari piuttosto che quello di cellule isolate.

Come modelli cellulari, sono state usate le vescicole “giganti” (GVs), le quali, grazie alle loro grandi dimensioni (1-100 micron), permettono la loro osservazione diretta mediante microscopia ottica e a fluorescenza.

Le vescicole giganti sono state prodotte con il metodo dell’inversione di una emulsione. Questo metodo è stato selezionato per la sua versatilità e per il fatto che permette di raggiungere alte rese di intrappolamento di vari soluti. Tuttavia, questo metodo era stato appena scoperto quando questo lavoro di dottorato di ricerca fu avviato, e sostanzialmente non era disponibile alcun protocollo standard. Abbiamo quindi, come primo passo, delineato un protocollo sperimentale che permettesse la produzione di vescicole giganti in modo altamente riproducibile e con alte rese (10000 GVS/ul). E’ stato possibile ottenere GVs con una soluzione interna diversa da quella esterna, e intrappolare una vasta gamma di soluti idrosolubili nel loro nucleo acquoso, come calceina (una piccola molecola solubile), e RITC o FITC-destrano (polisaccaridi), alloficocianina (APC), ficoeritrina (PE) (proteine). Risultati preliminari hanno esteso questo metodo per l'intrappolamento degli acidi nucleici e degli enzimi.

Al fine di creare un modello di colonie primitive abbiamo sfruttato l'attrazione elettrostatica, una forza fisica molto semplice e a lungo raggio, che deve aver avuto un ruolo chiave anche nei tempi primitivi. Come modello di peptide abbiamo impiegato la poli(arginina) (PLA), un policatione, per attivare la aggregazione di GVs anioniche (POPC:oleato 1:1). Immediatamente dopo l'aggiunta di PLA, si osserva che le GVs si spostano le une verso le altre, e si legano formando “colonie” di GVs di varie dimensioni.

Il processo di formazione di colonie è stato caratterizzato cambiando le variabili che ne influenzano la formazione. Il primo passo è stato quello di testare la concentrazione soglia di PLA capace di indurre aggregazione. Il secondo passo è stato quello di verificare se la formazione di colonie dipendeva dal numero di GVs inizialmente presenti. Grazie a questi esperimenti, siamo in grado di osservare il

processo di aggregazione delle vescicole in colonie in modo riproducibile e controllata.

Indipendentemente dalla quantità di PLA aggiunto o dal numero di GVs inizialmente presenti, è stato sempre possibile rilevare nuove GVs, in ragione del 1-5% rispetto al numero totale, che ne derivano senza dubbio dalla fusione di due o più GVs. Questo primo importante risultato conferma che la formazione di colonie determina un aumento di complessità, e fornisce alla colonia un vantaggio rispetto alla GVs individuali. Tale vantaggio è dato dall'aumento della complessità molecolare che si ottiene dal mescolamento di più soluti inizialmente separati, rendendo possibile quindi la loro interazione. Ulteriori vantaggi delle colonie GVs in relazione a singole GVs libere risiede nella loro resistenza al flusso, e nei confronti di stress osmotici. Inoltre, le colonie GVs può crescere integrando nuove GVs.

In conclusione, questo lavoro ha cercato di proporre una nuova visione per quanto riguarda i fenomeni alla base dell'origine della vita. E' la prima volta che si passa da uno scenario focalizzato su una unica cellula a quello di una comunità cellulare. Una colonia di individui che cooperano in modo sinergico è in grado di acquisire vantaggi selettivi rispetto ad individui isolati, e quindi evolversi e raggiungere un maggiore livello di complessità attraverso lo scambio di soluti, anche a causa di una maggiore stabilità fisica. E' verosimile pensare che questo nuovo modello di cellule primordiali, caratterizzate da una visione comunitaria, sia in grado di simulare più realisticamente i primi passaggi dell'evoluzione cellulare.

Abbreviations

ADF-cofilin cofilin derivatized with actin depolarizing factor
Alexa Fluor 350 Alexa Fluor probe (excitation 346 nm, emission 442 nm)
APC allophicocyanin
BSA bovine serum albumine
BSA-RITC Rhodamine labeled BSA
BSA-TRITC Tetramethylrhodamine labeled BSA
DABCO 1,4-diazabicyclo[2.2.2]octane
DDAB didodecyl-methyl-ammonium-bromid
DMPC 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine
DOGS-NTA-Ni 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)imidodiacetic acid)succinyl, nickel salt
DOPC 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine
DOPS 1,2-dioleoyl-sn-glycero-3-phosphatidylserine
DOTAP dioleoyl trimethylammonium propane
DPPC 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine
DTT dithiothreitol
EggPC egg yolk phosphatidylcholine
EGTA ethylene glycol tetraacetic acid
FITC-dex Fluorescein isothio-cyanate-Dextran
GVs Giant Vesicles
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
POPC 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine
POPG 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol
POPS 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylserine
PUCE 1-palmitoyl-2-undecylcarnitine-ester chloride salt
RITC-dex Rhodamine B isothio-cyanate-Dextran
TBS tris buffered saline
Tris HCl Trizma base, minimum
VVCA-His N-WASP fragment WWA with an N-terminal his_6 tag

Chapter 1. Introduction

1.1 The Origin of life

Human beings, during the course of history, has always placed the existential questions like where do we come from? What is the purpose of our lives? What comes after death? And – above all, "*what is life?*" Which are the laws that govern nature? How life originated on Earth?
During the course of centuries, the human curiosity and the desire to provide answers to those questions, has stimulated the work of philosophers and scientists, but to the present day, we do not have yet a clear understanding of what is life and how it originated on the Earth. What is the scientific view of the origin and definition of life?

1.2 Origin and definition of life

In primitive times, till the born of experimental scientific approach, the idea of spontaneous generation was generally accepted. We have to wait the XVIII and XIX century, when many scientists performed experiments to refute the spontaneous generation of microorganisms in solutions of organic substances. Only thanks to Louis Pasteur, who in 1862 published his investigations on spontaneous generation and in a series of brilliantly executed experiments, which left no room for scepticism, that it was demonstrated the utter impossibility of the formation of microorganisms in various infusions and solutions of organic substances.
No new notable research or theory on the subject appeared until 1924, when the Russian scientist Alexander Oparin reasoned that atmospheric oxygen prevents the synthesis of certain organic compounds that are necessary building blocks for the evolution of life. In his "*The Origin of Life*", Oparin proposed that the "spontaneous generation of life" that had been attacked by Louis Pasteur, did in fact occur once – in the primitive Earth, but it was later impossible because the conditions found in the early Earth had changed, and the presence of living organisms would immediately consume any spontaneously generated organism. Oparin argued that a "primeval soup" of organic molecules could be created in an oxygen-less atmosphere through the action of sunlight.

Many modern theories of the origin of life still take Oparin's ideas as a starting point. Around the same time, J. B. S. Haldane suggested that the Earth's pre-biotic oceans (very different from their modern counterparts) would have formed a "hot dilute soup" in which organic compounds could have formed.

The current scientific views on the origin of life on Earth take as starting point the Oparin-Haldane hypothesis, and most research has been carried out in the twenty century along this direction.

Under this umbrella, there have been found a wide array of disparate discoveries and conjectures such as the following, listed in a rough order of postulated emergence:

1. Some theorists suggest that the atmosphere of the early Earth may have been chemically reducing in nature, composed primarily of methane (CH_4), ammonia (NH_3), water (H_2O), hydrogen sulfide (H_2S), carbon dioxide (CO_2) or carbon monoxide (CO), and phosphate (PO_4^{3-}), with molecular oxygen (O_2) and ozone (O_3) either rare or absent.
2. In such a reducing atmosphere, electrical activity can catalyze the creation of certain basic small molecules (monomers) of life, such as amino acids. This was demonstrated in the Miller-Urey experiment by Stanley L. Miller and Harold C. Urey in 1953.
3. Fatty acids as well as phospholipids (of an appropriate length) can spontaneously form closed shells surrounded by lipid bilayers, i.e., *lipid vesicles* (also called *liposomes*), which are structurally similar to living cells.
4. A fundamental question is about the nature of the first self-replicating molecule(s). Since replication is accomplished in modern cells through the cooperative action of proteins and nucleic acids, the major schools of thought about how the process originated can be broadly classified as "proteins first" and "nucleic acids first". Here the views become less convincing, even if the standard paradigm of the RNA-first systems (the so-called RNA world) is currently presented in every biology textbook. This view, however, contrast with the experimental evidence of the quite difficult "prebiotic" synthesis of long RNA molecules (requested for catalysis, i.e., ribozyme species), and there is still no agreement on this aspect.

One of the consequences of the RNA-centred view of origin of life is that according to such view, the emergence of the first self-replicating molecule would correspond to the origin of life.
However, it is also well known that a single isolated molecule cannot be alive. Instead, life can be defined as a property of *molecular systems*, where reciprocal dynamic relationships and confinement in space are the two main ingredients.

In conclusion, the basic assumption is that life on Earth originated from inanimate matter by prebiotic molecular evolution, throughout a very long and lengthy series of steps of increasing complexity, often dominated by contingency. This leads up to the formation of self-reproducing protocells that were finally able to display biological autonomy, and start the ascent towards biological evolution and the construction of all living entities. The basic idea is namely that life developed by itself, without any transcendental help. It is fair to say that this is a hypothesis, as it has not been demonstrated yet.

The general concept of "life" becomes therefore associated to that one, more specific and realistic, of *cellular life*, where the compartmentation nature of the cell is one of the key features that allow the emergence of the living. This view finds the most elegant and powerful representation in the concept of autopoiesis.

1.3 Autopoieis and the Logic of Cellular Life

Cell's life is the starting point for the development of autopoiesis (from Greek *auto* = self, *poiesis* = production), introduced in the Seventies by the two Chilean biologists Humberto R. Maturana and Francisco J. Varela (Maturana and Varela, 1980). Autopoiesis does not concern the origins of life, it answer, instead, to the question "what is life", analyzing the living organism as it is – here and now – without questions about how it originates. In this sense, autopoiesis is a descriptive theory, and founds its strength on the analysis of organization of the processes that underlie the biochemistry of cell. Moreover, it can be applied as well to different hierarchical levels, from cells to societies, and also abstract concepts as cognition can be approached by the autopoietic theory; this advanced aspects, however, will be not discussed here (Luisi, 2003).

It is well known that even the simplest cell, such as a bacterium, actually consists in a maze of metabolic pathways (circular, controlled, regulated, etc.) involving interplay of nucleic acids, enzymes, and several chemical building blocks. When a cell is alive, the molecular components are continuously built and degraded, nutrients are taken up from the environment, and waste products are discarded. All components of a cell are subjected to turnover. The semi-permeable boundary is a key element of autopoietic view. It allows selective passage of elements in and out the cell, without releasing all components in the environment. However, despite these continuous transformations, which act on the molecular level, the cell – at an higher hierarchical level – maintains its identity. Under steady-state conditions, i.e., homeostasis, the cell regenerates, within its own boundary, all those chemicals that are being destroyed or transformed (boundary molecules included!). The cell, being a dissipative, thermodynamically open system, utilizes available nutrients from the environment and transforms them into components of the cell itself. To do so, cells do not need external commands, orders, or programs. All what is needed is already stored inside in form of organized reaction network. Therefore, a cell is a thermodynamically open but operationally closed system, and an autopoietic unit is a system that sustains itself thanks to an inner reaction network that generates the system's components (Fig.1).

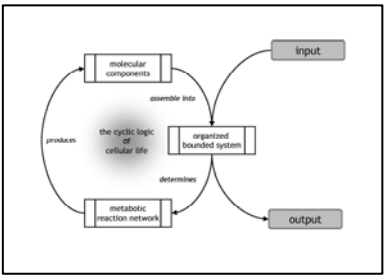


Fig. 1: The cyclic logic of cellular life. The cell – an autopoietic unit – is a self-organized bounded system that determines a network of reactions, that in turn produces molecular components, that assemble into the organized system, that determines the reactions network, that …

It is clear, therefore, how autopoiesis is so strongly connected with the definition of life at the cellular level. In fact, the property of being alive belongs to the cell as a whole and not to single parts. In this respect, autopoiesis is clearly related to the compartment approach that is so important in origin of life scenarios.

The theory of autopoiesis stimulated some experimental research programs, like those related to the self-reproduction of vesicles, micelles and reverse micelles (Stano and Luisi, 2010).

Ultimately, the whole approach to the construction of synthetic or semi-synthetic cell can be ascribed to the theory of autopoiesis.

1.4 The "Minimal Cell" project

Thanks to the concepts in autopoiesis, we have a theoretical framework for exploring, experimentally, the issue of the origin of cellular life from separated components. In particular, it is evident that we are not interested in create models of modern cells, due to the fact that they are firstly very complex, and second we need to capture the essence of life, the minimal requirements for life, also considering that primitive cells could not have been as complex as modern ones.

In fact, even the simplest living cells existing on Earth have several hundred genes, with hundreds of expressed proteins, which, more or less simultaneously, catalyse hundreds of reactions within the same tiny compartment, an amazing enormous complexity. This is the result of billions of years of evolution in which a series of defence and control mechanisms, redundancies and metabolic loops was developed.

The definition of life is notoriously a very controversial subject, however most scientists would be satisfied with a general, cellular-level, and very comprehensive definition of this type: life corresponds to the simultaneous implementation of three basic properties: self-reproduction, metabolism (or more precisely self-maintaining), and evolvability. All these considerations contribute to define an ambitious experimental project, called "The Minimal Cell" project which aims to build a synthetic or semi-synthetic cell in the laboratory, which is living but with a much lower degree of complexity than our modern cells, and having the minimum number of components and sufficient to define "alive", or a cell capable of self-maintenance (metabolism homeostasis), self-reproduction, and having the ability to evolve.

In the top-down approach we would like to build a minimal cell model by let molecules and lipids self-assemble in form of a compartment and study of the occurrence of the molecular reactions therein. By molecular reactions we mean all these transformations that are required for sustain a minimal metabolism and self-reproduction. We can therefore imagine an experimental roadmap that starts from the control of compartment formation, to the study of

simple and later complex enzyme-catalyzed reactions inside lipid vesicles, to the more complex protein synthesis, DNA/RNA synthesis, and lipid synthesis. Finally, it will be required the concerted, simultaneous reproduction of internal components (proteins, DNA, RNA) and of the lipid shell, in order to achieve a self-reproductive dynamics.
In the research group that hosted my PhD work, the Minimal Cell project is approached from several complementary directions. The EU-FP6 Synthcells project deals with the engineering of synthetic and semi-synthetic "modules" aimed at the final construction of minimal cells (Luisi *et al*., 2006); whereas in the Human Frontier project much emphasis is given to the biomolecular approach and protein synthesis by purified components entrapped into liposomes (the "pure-system" kit, developed in Japan by Prof. Takuya Ueda and co-workers (Shimizu *et al*., 2001)).
In both cases, the central ingredient for the study of minimal cell is the notion of compartment and the formation of a structure that contains all necessary components for functioning.
Here we see the first important connection with origin of life scenarios. When real systems are considered, what is the probability of the formation of a structure containing all needed components?
A recent work calculated that the probability of co-entrapping tens of different compounds in the same compartment is vanishing small, especially for solutes at low concentration and small vesicle size (Souza *et al*., 2009). It follows that additional mechanisms, like fusion or solute exchange, could have played a role in origin of life scenarios, so that the interaction among different compartments – very often neglected – could have had a key role in the developments of early protocells.

As it will be discussed later, this theme is the central idea discussed in this thesis: how can we depict realistic mechanisms that would lead to the achievement of a functional (yet minimal) protocell starting from simpler compartments and taking into account the adverse probability of solutes co-entrapment in a single vesicle? In other words, when we consider the study of minimal cell and their role in the origin of life, it is correct to consider them individually, looking at the "single-compartment" level, or is it more realistic to consider how vesicles in a population, thanks their mutual interactions can *cooperate* and *collectively* bring about to jumps along the route from simple to complex systems?

In the next paragraph, the liposome model will be introduced, by shortly discussing their properties, formation and relevance in origin of life. More in particular, since this thesis deals with giant vesicles (GVs), i.e., vesicles having micrometric dimensions, the final paragraph will provide a short overview of current methods of GVs formation, with particular attention to one of the newest method, the "emulsion inversion" one, also known as "droplet transfer", which is very promising in Minimal Cell research.

1.5 Lipid vesicles (liposomes)

The term "vesicle" (from the Latin *vesicula, -ae*, small bladder) is used to designate a particular type of compartment, formed an aqueous medium. The interior of a vesicle is a small aqueous volume and the boundary of each vesicle is constituted by one (or a few) thin layer(s), also called membrane, composed of amphiphilic molecules.

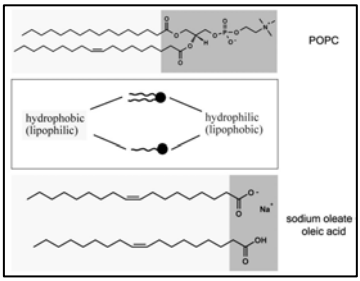


Fig. **2:** Molecular structures of POPC (a double-chain amphiphile) and sodium oleate/oleic acid (single-chain amphiphiles).

The amphiphiles are molecules contain hydrophilic and hydrophobic regions, typically arranged in head-tail geometry. Vesicles can be formed by single-chain or double-chain amphiphiles; fatty acids and phospholipids are examples of the first and second class, respectively (Fig.2).

The amphiphiles in a vesicle membrane are arranged in such a way that the hydrophilic parts are in contact with the aqueous medium, while the hydrophobic parts associate to form the interior in each layer (Fig.3).

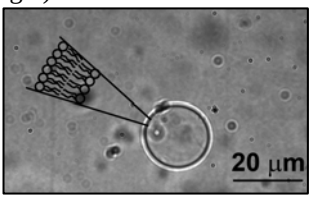


Fig. 3: Bright field image of POPC GV. The drawing shows the arrangement of amphiphilic molecules at the membrane Size bar = 20 μm

Vesicles in which the membranes are constituted from lipids present in biological membranes (for instance phospholipids such as POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) are often called

lipid vesicles or more frequently liposomes, or also artificial vesicles. Finally, vesicles prepared from synthetic surfactants, which are chemically different from naturally occurring amphiphilic lipids, are also called (synthetic) surfactant vesicles

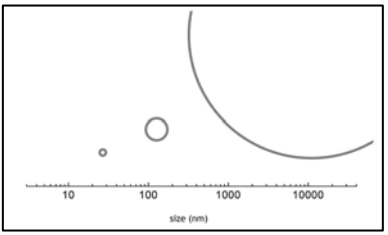


Fig. 4: SUVs, LUVs and GVs along a logarithmic size axis (vesicles drawn not to scale).

Vesicles are classified according to their size. There are small unilamellar vesicles (SUVs) with a size around 30-40 nanometers; large unilamellar vesicles (LUVs) with a size of hundreds nanometers and also Giant Vesicles (GVs) which have a size more than one micrometer, and therefore can be directly visualized by optical microscopy (Fig.4). For the latter reason, their use in Minimal Cell research is quite interesting, because it allows the observation of these lipid compartments, as well as their morphological transformations and internalised chemical reactions.
GUVs, as well as LUVs and SUVs are aggregates that are usually not at a "true" thermodynamic equilibrium, but rather in a kinetically trapped state. The energy barrier often is so high that the "true" thermodynamic equilibrium of the system cannot be reached easily. Once formed, the vesicles might remain as a kinetically stable system for an extended period of time, which can be hours, days or even weeks.

1.6 Giant Vesicles (GVs): Preparation and application

Giant vesicles (GVs) are considered a valid model for building minimal cell because they have similar size of living cells, in the range of about 1–100 um, and they can be studied with conventional light microscopic techniques. The limited resolution of light microscopy, however, does not allow to readily distinguish unilamellar from oligolamellar vesicles, except if unilamellarity is confirmed by corresponding bending elasticity measurements, or determined with fluorescence assays (Akashi *et al*., 1996). Depending on the experimental conditions, such as the osmotic pressure difference between the vesicle's interior and exterior, giant vesicles can be spherical or non-spherical.

A giant unilamellar vesicle (GUV) resembles the basic compartment structure of all biological cells, in the sense that the vesicle membrane is a mimic of the self-closed lipid matrix of the plasma membrane. To illustrate the geometric properties, a spherical unilamellar giant vesicle with a diameter of 50 um and a membrane thickness of 4 nm is first considered; 4 nm is the approximate thickness of a hydrated POPC bilayer. It is astounding that a membrane that is only 4 nm thin can separate the inside of such a large object from the external aqueous solution. If extrapolated to sizes with which humans are more familiar, the relationship between vesicle diameter and membrane thickness becomes more obvious. Taking instead of a 50 um giant vesicle a balloon with a diameter of 50 m, this balloon would have a skin with a thickness of only 4 mm.

Due to their cell-mimicking characteristics, GUVs currently are intensively studied in different areas of biomimetic chemistry, biomembrane physics and in the field of artificial cell synthesis. In many studies that focus on mimicking biological membranes, for example by reconstituting membrane components in giant vesicle bilayers. One of the most obvious applications of giant vesicles is their use as simple model systems for studying certain physicochemical properties of biological membranes. Examples include mechanical properties of the entire vesicle or of the membrane, lipid domain formation, lipid dynamics, membrane growth, budding, fission and membrane fusion.

A further application of giant vesicles is in the field of membrane protein research. The prerequisite for such studies is that the membrane protein of interest can be reconstituted in a giant vesicle membrane. Classical procedures allow the reconstitution of membrane proteins in submicrometer-sized vesicle membranes, typically by the detergent depletion method. However, this method is not directly applicable to the formation of giant vesicles. Girard *et al.* (Girard *et al.*, 2004) described a procedure for the successful reconstitution of the sarcoplasmic reticulum CaII-ATPase and the H^+-pump bacteriorhodopsin in giant vesicle membranes.

There are several advantages of using giant vesicles instead of submicrometer-sized vesicles, for example in studies of the effect of antimicrobial substances (for example, antimicrobial peptides) on phospholipid membranes. If a suspension of SUVs is used, only average observations are possible. Reactions occurring within or on the surface of single vesicles cannot be measured with SUVs.

Studies with SUVs and GUVs give complementary information. By using GUVs it was for example possible to study details of vesicle membrane fusion and fission; this provided unique insights into the two processes. Furthermore, the use of GVs allowed a direct visualization and quantitative analysis of the pore formation in phospholipid membranes by the peptide magainin-2.
Perhaps one of the most fascinating and challenging fields of application of giant vesicles is the research that aims at preparing systems that have at least some of the basic features of living cells.
Currently, the expression of proteins inside vesicles is the state-of-the-art in this field of research. From a theoretical point of view, this is of relevance, because the activity of a synthesized protein might lead to complex reaction dynamics in the system, with consequences at the macroscopic level, such as internal enzyme-mediated lipid synthesis with consequent vesicle growth, or vesicle-vesicle interaction mediated by membrane proteins, or uptake of externally added nutrient or signals by membrane receptors, and many other examples. In general terms, the expression of functional proteins inside GVs allows the experimenter to build "from inside" a complex bioreactor that mimics in great detail the basic behavior of cells. This approach, that can be defined as "learning by constructing" is typical of the new discipline of *synthetic or constructive biology*, which aims to construct novel biological parts, devices and systems to perform useful functions or to understand at the most basic level the mechanism of certain biochemical patterns (the frequently cited motto is "What I cannot create, I do not understand" by Richard Feynman).
Due to their large size, giant vesicles are not suitable for drug delivery applications, but they might find applications in the field of biosensors, for example to detect permeabilizing toxins or xenobiotics.

1.7 GVs: main preparation methods

Historically, one of the first methods for the preparation of giant vesicles was described by Reeves and Dowben in 1969. It was a controlled hydration of a thin dry film of egg yolk phosphatidylcholines (egg PC) deposited on the glass surface on the bottom of a flat-bottomed 2 L Erlenmeyer flask. The lipid film was allowed to swell for two hours or more, and this led to the formation

of giant thin-walled vesicles. Even slight agitation during the swelling period tended to diminish the yield of thin-walled vesicles.
When the lipid film is hydrated, a continuous swelling of the bilayers occurs, more and more water molecules penetrate between the bilayers, into the interlamellar space. The result is that giant vesicles slowly grow in volume and finally detach from the surface.

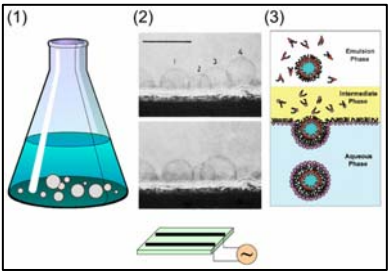


Fig. 5: Schematic drawing of three important GVs production methods (1) natural or gentle swelling; (2) electroswelling; (3) emulsion inversion (or droplet transfer).

In addition to the controlled hydration method, there are several other methods to obtain relatively homogeneous and mainly uni- or oligolamellar giant vesicles. They cannot be discussed here in details for reasons of space. There are however three methods that are important in Minimal Cell research: (1) the natural swelling; (2) the electroswelling; and (3) the emulsion inversion (also known as droplet transfer) (Fig.5).

1.7.1 The Natural Swelling method (Fig. 5.1)

The principle of the method is the one just described. It consists of the controlled hydration of (dry) lipids deposited on a solid surface, mainly glass, as originally developed by Reeves and Dowben (1969). This method is also known as spontaneous swelling, natural swelling or gentle hydration method. The method is usually particularly successful for the preparation of GUVs from samples containing charged lipids. Furthermore, it is important to note that the hydration has to be carried out in the liquid-disordered state of the bilayers, that is, at a temperature above T_m, corresponding to the so-called solid-ordered (s_o)/liquid-disordered (l_d) or gel/liquid-crystalline main phase transition temperature.

1.7.2 The Electroswelling method (Fig. 5.2)

In 1986, Angelova and Dimitrov published a study concerning the effect of externally applied electric fields on the hydration of lipids deposited from an organic solution on a conductive glass surface (indium tin oxide (ITO) coated glass), or on platinum wires.

Depending on the experimental conditions, that is, type, composition and amount of lipids used, applied electric field parameters, hydration medium, rather homogeneous giant unilamellar vesicles can be obtained in aqueous solution or buffer solutions of low ionic strength. The method is known as electroformation or electroswelling method (Angelova and Dimitrov, 1986).
Electroformed vesicles remain connected to the residual lipid film on the electrode on which they are formed. This might be an undesired property or an advantage, particularly if individual vesicles shall be punctured with a micro-needle. The electrode provides a back pressure and the connected vesicles do not move away easily during micromanipulation.
The spontaneous swelling and electroformation methods generally do not allow efficient encapsulation of large water-soluble molecules (e.g., enzymes) or charged compounds during the formation process because the molecules to be encapsulated have to somehow move below the outermost layer of the deposited lipid film; this is difficult due to slow transbilayer movements of large or charged molecules. Encapsulation is, however, possible after vesicle formation by microinjection, puncturing individual vesicles with micro-needles.

1.7.3 The Emulsion Inversion (or droplet transfer) method (Fig. 5.3)
If a (stable) w/o emulsion can be prepared from an oil and a bilayer-forming lipid, this w/o emulsion can be used as a starting system for the preparation of giant vesicles. The w/o emulsion is poured onto a two-phase system consisting of a lower aqueous phase and an upper oil phase containing a bilayer-forming lipid. If the two phase system is first preincubated for a while prior to the addition of the w/o emulsion, the interfacial region between the two phases will become saturated with lipids, and form a monolayer in which the hydrophilic head groups of the lipids are in contact with the lower aqueous phase and the lipophilic chains protrude into the upper oil phase containing excess lipid. Because water is heavier than the oil used, the water droplets of the upper w/o emulsion tend to move from the w/o emulsion toward the oil/water interface and finally through it into the lower aqueous phase where giant vesicles are formed. If spontaneous water droplet migration does not occur, or only slowly proceeds, centrifugal forces can be applied to force the water droplets to move from the upper w/o emulsion through the interface into the lower aqueous solution.

In another approach, again starting with a lipid-stabilized w/o emulsion, Tan et al. reported that giant vesicles can be formed from an initial w/o emulsion stabilized with a phospholipid by simple addition of this w/o emulsion to an ethanol/water mixture in a microfluidic device (Tan *et al*., 2006).

With classical preparation methods (as the gentle hydration) it is possible to prepare GVs with various types of lipids, unilamellar and in large numbers, but the entrapment of large amounts of solutes within the aqueous core is not easily done, especially for macromolecules. In contrary, by the method of w/o emulsion, it is possible to entrap solutes with high efficiency. Therefore, this formation method could be very useful when a large number of valuable solutes such as DNA, RNA and proteins must be co-entrapped in GVs.

Advantage and disadvantage of the three methods are summarized in Table 1.

Tab. 1: Some of the advantages and disadvantages of the different methods for giant vesicle preparation.

	Natural swelling	Electroswelling	Emulsion inversion (droplet transfer)
Advantages	Simple; no need of special equipment; mild conditions; physiological conditions can be used; use of charged lipids possible	Control of hydration process by adjusting electric field parameters; high reproducibility; giant vesicles formed are uniform, unilamellar and spherical; the vesicles are attached to the electrode which facilitates microinjection	No need of special equipment; possibility of forming vesicles with asymmetric bilayers; control of vesicle size by droplet size in the w/o emulsion; high encapsulation efficiency; high yield of vesicles
Disadvantages	Giant vesicle formation difficult to control (highly dependent on experimental conditions during hydration and dependent on lipid film thickness); difficult if only uncharged lipids are used; low encapsulation efficiency	Special equipment required (electrodes built in an observation chamber and an AC or DC device); no giant vesicle formation if the lipid deposit contains too many charged lipids; low yield of vesicles; low encapsulation efficiency; possible oxidation of polyunsaturated lipids	Possible presence of oil in the vesicle membrane; size range only up to a few micrometers; initial w/o emulsion might be heterogeneous

1.8 The emulsion inversion method as a convenient way to prepare GVs for minimal cell research (Table 2)

The method of GVs formation from water-in-oil emulsion, for the first time proposed by Weitz and coworkers in 2003, although conceptually very simple, not always gives good results, in terms of the number of GVs obtained and for the morphology of vesicles. The authors describe a number of technical limitations that restrict the use of this method. This technique relies on the formation of a stable inverted emulsion and on the passage of the emulsion droplets through a second interface to make the bilayer. Unfortunately, both of these steps create problems when lipids are used and limit the generality and practicality of this technique. The problems originate from the use of lipids in the role of surfactants. First, lipids form large aggregate structures in both oil and water and hence do not stabilize the interface of the emulsion as well as a traditional surfactant can; this severely limits the effectiveness of the emulsification and restricts the concentration of water that can be emulsified. Second, lipids adsorb very slowly to the interface and require extended periods of time to fully cover the surface. Third, lipids at the interface between oil and water apparently undergo spontaneous emulsification; this seems to result in a strongly preferred drop size, making it exceedingly difficult to produce drops of arbitrarily controlled sizes. Fourth, the slow equilibration of the interface makes it difficult to replenish the second interface as the emulsion drops pass through it; this severely limits the efficiency of the formation of the vesicles, particularly as the size of the droplets increases (Pautot *et al.,* 2003a).

Despite these limitations, the authors were able to entrap proteins, such as G-actin, and to obtain the polymerization reaction inside GVs of phospholipid vesicles. In another work, they report the formation of GVs with an asymmetric membrane (inner leaflet different than outer leaflet) by using POPC and POPS (Pautot *et al.*, 2003b).

The following year (2004), Noireaux and Libchaber at the Rockefeller University, constructed a cell-like model capable of express the enhanced green fluorescent protein (EGFP), by co-entrapping a cell extract of *E. coli* and the plasmid codifying for the EGFP inside GVs made of egg-lecithin (Noireaux and Libchaber, 2004).

Between 2006 and 2009, a group of Japanese researchers coordinated by Yoshikawa, deeply investigated the method of droplet transfer, and produced 5 scientific articles. They have studied in detail what happens at the water/oil interface, using a special microscope slide, consisting of a micro-chamber where they have reconstituted the two-phases system characteristic of the method. Using small volumes, they observe the crossing of water drops in oil through the interface and the spontaneous formation of giant vesicles in the aqueous phase driven only by gravity (Yamada *et al.,* 2006).
They showed, in their first articles, that water droplets spontaneously transfer from the oil phase to the water phase, and that, stable liposomes with controlled size and composition can be prepared in large quantity (Yamada *et al.,* 2007). Thereafter, they were able to obtain GVs with an asymmetric membrane and lipid raft micro-domain (liquid-ordered phase) (Hamada *et al.,* 2008). Finally, they have entrapped proteins at high concentration inside the GVs (Takiguchi *et al*., 2008), and studied the effect of osmotic stress using sucrose and KCl solutions at various concentrations as outer buffers (Ohno *et al.,* 2009).
In the articles cited above, the authors use the same slide with microchamber, and although many GVs are formed, they remain adhering at the water/oil interface, without being released in solution.
In 2008, Whittenton *et al.*, have been able to generate GVs with an asymmetric membrane and to entrap inside fluorescent oligoDNA, and other fluorescent markers. Vesicles produced have been found in the solution like in the early articles of Pautot *et al*., and Noireaux and Libchaber (2004). The authors use the centrifugation to accelerate the crossing of water droplets through the interface, obtaining less then 20% yield in terms of solutes entrapment (Whittenton *et al.,* 2008).
Cecile Sykes's group, in 2009, has been able to obtain GVs containing a concentrated salt solution containing the protein G-actin. The external environment is a concentrated salt solution where the nutrients are present. After the formation of vesicles alpha-hemolysin has been added, an extracellular protein secreted by *Staphylococcus aureus*, which assembles into a ring structure on membranes and forms transmembrane pores to render liposomes selectively permeable for nutrients. Only at this point the authors

observed the reaction of polymerization of G-actin (Pontani *et al.*, 2009).
Also in 2009, Nishimura *et al.*, used fluorescence flow cytometry, comparing the characteristics of GVs obtained by different methods of formation. GVs obtained with the method of water in oil emulsion have a thin lipid membrane and a more regular morphology than other formation methods (Nishimura *et al.*, 2009).
Finally, Kubatta and Rehage have studied the effect of different oil phases and of different kinds of lipids or surfactants. Changing these parameters, it has been possible to get GVs of various sizes and with different stability. The authors manage to prepare GVs with diameters in the order of magnitude of millimeters (Kubatta and Rehage, 2009).
In table 2 there is a list of the experimental conditions described in above-cited articles in which GVs have been produced by the method of water in oil emulsion.

Table 2. Review of the published experimental conditions involving the emulsion inversion method

ID	Lipids used	Organic phase	Inner Buffer	Outer Buffer	Interface incubation time and volume
1	eggPC; POPC; POPS; DOPS (alone or mixture) 0.05-0.5 mg/ml	dodecane; squalene	100 mM NaCl and 5mM Tris pH = 7.4	100 mM NaCl and 5 mM Tris pH = 7.4. (3 ml)	• 30 min (charged lipids) up to 90 min (zwitterionic ones) • 2 ml
2	POPC and POPS (asymmetric vesicles) 0.05 mg/ml	dodecane	100 mM NaCl and 5 mM Tris pH 7.4	100 mM NaCl and 5 mM Tris pH 7.4 (3 ml)	• 2–3 h • 2ml
3	egg lecithin 5mg/ml	mineral oil	*E. coli* extract	feeding solution (buffer of *E. coli* extract).	no interface
4	egg PC DOPC 0.1-1 mM	mineral oil	water	---	• 2 h • n.d.
5	DOPC 1, 2, and 5 mM	mineral oil	---	water (10 ul)	---
6	egg PC (0.35 mg/ml) – 1 mM; DOPC (0.1-1 mM); DOPC/DPPC/cholesterol 3/3/4 molar ratio 0.1mM	mineral oil	0.1-1 M sucrose	0.1-1 M glucose (10 ul)	• 2 h • n.d.
7	EggPC 0.5 or 1.0 mM	mineral oil	25mM imidazol-HCl, 5mM $MgCl_2$, 50 mM KCl, and 10 mM DTT in the presence or absence of 1 mM EGTA pH 7.5	25 mM imidazol-HCl, 5mM $MgCl_2$, 50 mM KCl, and 10 mM DTT in the presence or absence of 1 mM	---

				EGTA (10 ul) pH 7.5	
8	DMPC, POPC, DOTAP 0.13-1.0 mM	dodecane, mineral oil or squalene	TBS (100 mM NaCl and 5 mM Tris base) pH 7.4	TBS (100 mM NaCl and 5 mM tris base) pH 7.4.	---
9	EggPC/DOGS-NTA-Ni/cholesterol of 95:5:0 or 58:5:37 0.5 mg/mL.	mineral oil	0.1 mM $CaCl_2$, 10 mM HEPES (pH 7.6), 100 mg/mL dextran, 0.2 mM ATP, 6 mM dithiothreitol (DTT), 0.13 mM DABCO, 280 mM sucrose.	10 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 2 mM ATP, 6 mM DTT, 0.13 mM DABCO, 275 mM glucose, and 0.5 mg/mL casein.	• n.d. • 30 ul
10	Asolectin	dodecane, tetradecane, hexadecane, olive oil	10–20 wt.% sucrose, Tris buffer	100 mM NaCl and 5 mM Tris	---
11	POPC, POPG, and cholesterol at molar proportions of 83:0:17 or 75:8:17	liquid paraffin	150 mM sucrose, 350 mM glucose,	500 mM glucose (400 ul)	---
12	eggPC (0.5 or 1.0 mM)	mineral oil	water	sucrose or KCl, 10 uL	---

Table 2. (continued)

ID	W/O emulsion preparation, % and volume	Centrifugation	Size and number of GVs	Entrapped solutes	Reference
1	• gentle stirring, shear using a mixer, extrusion, and sonication • 0,5-1% • 0,1-1ml	120g/5-10 min.	• 1-4 um • n.d.	Dextran (10kDa) 1mg/ml in an aqueous buffer (0.1 M KCl with 10 mM Tris, pH 8; G-actin 5-15 um (polymerization reaction)	Pautot et al. 2003 (1)
2	• gently stirring the mixture with a magnetic stir bar for 3 hours • 0,5% • 100ul	120g for 10 min.	• <1 um • n.d.	---	Pautot et al. 2003 (2)
3	• gentle vortex for a few seconds • 0,5% • 50ul	no parameters	• 1-10 um • few hundreds vesicles	BSA-TRITC and fluorescein-12-UTP, A cytoplasmic extract (100 mg/ml protein, 50–100 mM salts, and 10–20 mM ions).	Noireaux and Libchaber, 2004
4	• pipetting up and down with a micropipette • 5%	no centrifugation	• 10-100 um • large quantity	---	Yamada et al. 2006
5	10 uL	no centrifugation	• 1-100 um • n.d.	---	Yamada et al. 2007
6	• tapping • 5% • 5ul	no centrifugation	---	---	Hamada et al. 2008

7	• pipetting • 5%	no centrifugation	---	200µM F-actin; heavy meromyosin (HMM)	Takiguchi et al. 2008
8	sonication and then extrusion	centrifuged at 115g for 30 min	a) dodecane • 8680 GVs/mm^2 • 13.9% entrapment b) squalene • 13006 GVs/mm^2 • 18.2% entrapment	fluorescently tagged DNAoligo (0.05-0.1 mM) and/or Alexa Fluor 350 salt (0.57-0.72 mM)	Whittenton et al. 2008
9	• pumping • 0,5% • 50 ul	100 x g for 12 min then 350g for 8 min	• 1 to 8 um • n.d.	0.12 mM for Arp2/3, 50 nM for gelsolin, 2 mM for ADF-cofilin, 1 mM for profilin, 6.5 mM for G-actin (including 20% fluorescently labelled actin) and 0.64 mM VVCA-His	Pontani et al 2009
10	---	No centrifugation	• 3-5 mm • n.d.	methyl blue	Kubatta and Rehage, 2009
11	• vortex 10s • 10% • 400 ul	18 000 g (14 000 rpm) for 30 min at 4 °C	---	allophicocyanin (APC)	Nishimura et al. 2009
12	• pipetting • 5% • 10 ul	No centrifugation	---	---	Ohno et al. 2009

Chapter 2. Aim of the work

As mentioned in the Introduction, investigations on the origin of early cells are currently performed by several group. In particular, one of the open questions concerns the steps that are required to achieve functional primitive cells starting from separated compartments. This aspect is discussed in terms of encapsulation of several biomolecules in primitive compartments; lipid vesicles, and more in particular fatty acid vesicles are the most plausible candidates of primitive compartments. This has stimulated some experimental research projects, as the "Minimal Cell" one, which aims at the laboratory construction of cell models containing the minimal and sufficient number of molecules and capable of displaying the most basic features of living cells.
However, from the conceptual viewpoint, the current approach is based on the idea that a single primitive cell forms directly from separated components or that a complex metabolism developed within a single primitive cell (starting from a simpler encapsulated molecular mixtures). However, alternatively, and perhaps more realistically, it is also possible that cellular or pre-cellular systems are not the product of individual events, but as the outcome of a series of multiple interactions within a community of cell-like structures, resulting cumulative benefits. We may rephrase these views as an open question: did cellular life originate from individual compartments or from a community of compartments. Such questions has been recently debated in a Workshop dedicated to Origin of Life (San Sebastian, Spain, June 2009).

From these considerations comes the question of how to create and to study a primordial protocell community.
The aim of my PhD work is the experimental study of minimal cell-like systems, and to understand the nature of the processes that led to the development and evolution of cellular life.
There are two main goals of this work. Firstly, define and develop cell-model systems, and then propose a novel view about the possible important steps in primitive pre-cellular evolution, from the viewpoint of cell communities.

GVs are useful models for the study of cells. They are closed compartments with a lipid membrane boundary, which separates an

interior content that can be very different from the external environment. Thanks to their semi-permeable membrane, GVs can exchange solutes with the environment. Most importantly, GVs have size between 1 and 100 um, which allow their direct observation by light microscopy. Thanks to all these features, that make them comparable to the actual living cells, GVs are very valuable models for developing cell models for ambitious projects as the “Minimal Cell” one. The most promising method for GVs production is the recently reported “emulsion inversion” (or “droplet transfer”) method, which allows the preparation of GVs with high efficiency of solute entrapment. This method is therefore essential for the construction of Minimal Cells. However, this method is quite new and – as evident by the analysis of literature – is not easy to reproduce. When this work was started, not only the know-how for producing GVs with this method was not available in my hosting group, but also a very limited number of reports was published. Actually, our group started the development of this GVs technology simultaneously to other international groups.

The first goal in this study was to create GVs in large quantities, in a reproducible and controlled manner, and with several kinds of solutes entrapped inside.
This achievement is a major step forward in developing new methods for studying minimal cells, not limited to the current work – which would address more basic aspects, but more in general for constructing bio-reactors capable of sustaining minimal metabolism or be able to protein expression.

The second goal will then be the development of innovative systems for understanding whether and how GVs communities are formed, if they have an advantage in respect of single GVs, and if it is so, to know how to create and manipulate them in a controlled and reproducible way. This would represent an extension and an evolution about the studies on the origin of life and on the primordial cells, shifting the focus from individual compartments to GVs colonies in order to simulate a more likely reality of cellular life.
If this community model works, one could then expand it, and give a new vista on the study of GVs-based systems as model for primitive cell “ecology”, that is also a quite new concept in current research.

Although being a very ambitious goal, the work carried out in this thesis, might certainly help to advance in constructing cell model systems for studying the mechanisms underlying the development of cellular life.

Chapter 3. Results and discussion

3.1 Optimization of GVs production formed from a lipid-stabilized water in oil emulsion.

As stated before, the main goal of my PhD work is the experimental study of cell models at the aim of understanding in greater details the early steps of primitive cell development, within the so-called Minimal Cell project. At this aim, GVs will be used as standard cell model and – for the first time in the literature – it will be investigated the importance of intervesicle interaction as a way to progress along the path of functional cell formation, starting from simpler cellular or protocellular systems.

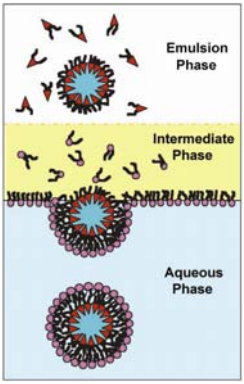


Fig. 6: Scheme of the emulsion inversion method. From Pautot *et al* 2003a.

We have chosen a preparation method that allows the formation of GVs with high entrapped yield. As noticed in the Introduction, Pautot *et al*. (2003), as well as Noireaux and Libchaber (2004) have reported on the conversion of w/o droplets into GVs. The method of preparing GVs starting from w/o emulsion droplets is extremely advantageous when bioreactors must be constructed, and more in general in our top-down synthetic biology approach. In fact, thanks to the total entrapment of water-soluble solutes in w/o droplets, it becomes feasible the construction of lipid based compartments containing a high number of diverse macromolecules and therefore build complex cell models, that we may call semi-synthetic minimal cells.

The method was introduced by Pautot *et al*. (2003) Pre-formed w/o droplets are centrifuged in a two-phase medium, so that they convert in GVs by crossing the macroscopic interface (see Fig.6).

The first step is the preparation of a water-in-oil (w/o) emulsion, which can be obtained by emulsifying a small aqueous volume in a lipid-containing apolar solvent mineral oil by means of mechanical forces (stirring, vortexing, shaking). This step can be describe in the following terms: the macroscopic water droplets, possibly containing solutes, is broken in millions of small droplets which are stabilized by lipids at the w/o microinterface, presumably forming a lipid

monolayer. In the absence of lipids, the emulsion is unstable and quickly returns to the state of two macroscopically separated phases.

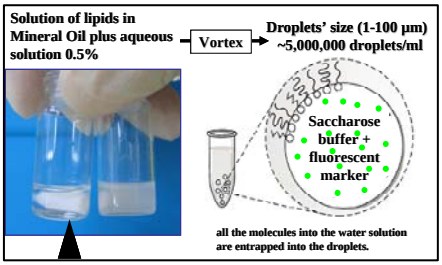


Fig. 7: Preparation of a water in oil emulsion. A big drop of water solution (it is indicated from the arrow) is broken in millions of small droplets which are stabilized by lipids that formed a monolayer.

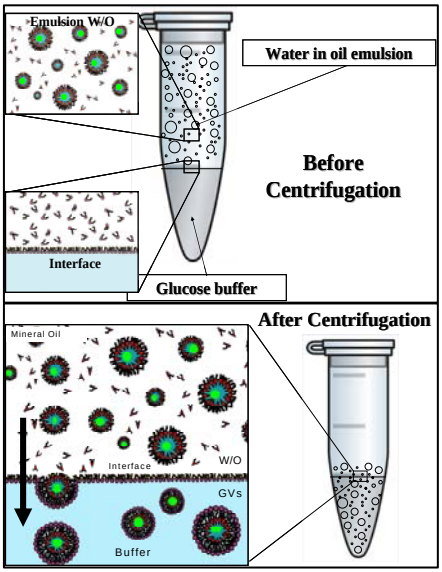


Fig. 8: Scheme of the emulsion inversion method. Before centrifugation, in the eppendorf there are three phases: water in oil emulsion, oil-water interface and an aqueous solution. After centrifugation, the water in oil droplets are transformed into GVs when they cross the oil-water interface.

The hydrophilic solutes which were present in the aqueous drop are therefore segregated into the w/o droplets, becoming entrapped – in the next step, into the GVs. Ideally, each w/o droplet is transformed into a GV. It is convenient to dissolve fluorescent probes so that the w/o droplets first and the GVs later can be observed, also quantitatively, by fluorescence microscopy. In order to collect the GVs after passage through the macroscopic oil/water interface, the solution used for preparing the emulsion must be denser that the aqueous solution placed below. This is achieved by using sucrose- vs glucose-containing buffers. Glucose and sucrose are two sugars that are compatible with all biomolecular systems we are interested in. Therefore, different densities are obtained by using sucrose in the "inner solution" (the aqueous solution used to make the emulsion, that will

become the internal GVs solution) and glucose in the "outer solution" (the aqueous solution used to collect GVs, that is the external GVs solution). In order to avoid osmotic stress, isotonicity is obtained by using the same sugar concentration. For example, 100 mM sucrose has a density of about 1.011 g/l, whereas this value is 1.005 in the case of 100 mM glucose. From the practical viewpoint, the w/o emulsion, prepared by emulsifying an aqueous solution of a fluorescent dye is poured above a lipid-containing oil layer that represent the "interface" phase. Below them, there is the aqueous phase where the GVs will be collected (Fig. 8). At the interface between the oil and water phases, the lipids have the polar head in the water solution and hydrophobic tail in the mineral oil. In order to collect GVs from w/o droplets, the eppendorf tube is centrifuged. The droplets are then converted into GVs when they cross the oil/water interface.

After some preliminary testing, we have firstly shown that method works also when the zwitterionic phospholipid POPC is used. GVs were heterogeneous with respect to size, namely between 1 to 50 micrometers (diameter), and in their aqueous core there was a fluorescent marker. However, despite the large amount of water in w/o droplets (summed up to 1 ul), a small number of GVs was initially observed, and – at the same time – the aqueous phase (the "outer phase") contained some of the fluorescent marker that was initially present in the w/o droplets. When a hydrophobic marker (rhodamine 6G) was added to the mineral oil solutions, and this solution was used to prepare a w/o emulsion from a calcein solution, it was possible to produce double-labeled GVs (Fig.9). The green fluorescence from the aqueous GV core and the red fluorescence from the GV membrane confirm the correct position of both fluorophores.

Initial experiment, although successful, showed that even if the "emulsion inversion" method is clearly extremely advantageous for minimal cell research, it is also very difficult to achieve. Indeed the authors of the articles do not explain in detail the experimental conditions, and, due to the novelty of this method, in literature there are only a very limited number of reports (see Introduction). Therefore, the first part of the work has been devoted to optimize the method of GVs production via "emulsion inversion". The goal was to obtain in reproducible way the maximal number of solute-filled GVs, by using different markers.

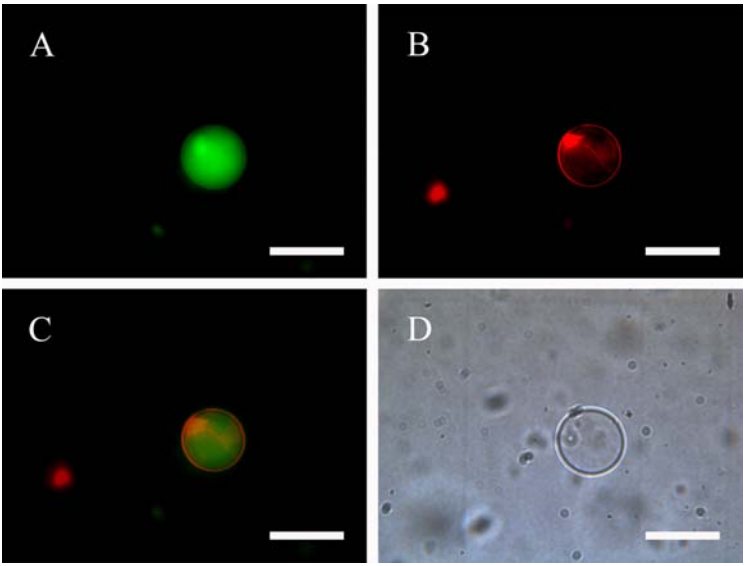


Fig. 9: Double-labeled POPC GV. (A) Calcein in the aqueous core; (B) rhodamine 6G on the membrane; (C) overlapping of channels (A-B); (D) bright field image. Size bar = 20 µm.

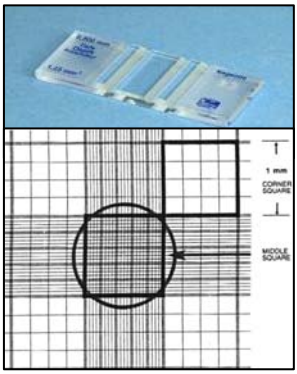


Fig. 10: Hemocytometer microscopic slide. This Particular kind of microscope slides has a grid with a defined size. It is usually used in Cell Biology to cell counting. In our case it was used to count GVs.

To quantify the effectiveness of GVs production (expressed in number of GVs/mL, i.e. numerical density) we used a hemocytometer, which generally used in cell biology to count cells (Fig. 10). The hemocytometer is loaded by a definite volume of GVs dispersion, and thanks to its calibrated grid it is possible to estimate the number of GVs by direct visualization at the microscope. GVs are very difficult to identify in bright field. They are almost invisible because they are not colored. So it is virtually impossible to count them directly under the microscope as it does in cell biology for the cells. But when GVs are produced by employing a fluorescent marker (trapped inside) it

become possible to see them by fluorescent microscopy, still using the hemocytometer. In our approach, we have collected and analyzed by a image analysis software (Image J) the number of GVs in each micrographs, and from this value the numerical density is easily calculated by taking into account the sample volume.
With this strategy, we were able to assess what were the best experimental conditions for the emulsion inversion method.

3.1.1. Effect of the lipids in w/o emulsion and interface: lipid mixture and "asymmetric" design

Firstly, GVs production was attempted by using the same lipid or surfactant in the w/o emulsion phase, and in the interface phase. We employed phospholipids like POPC, POPS, POPG, fatty acids like sodium oleate, and surfactant like DDAB and PUCE. In all cases, we got a very limited number of GVs (less than 100/ml). When POPC, POPG and POPS were used a few vesicles with sizes between 1 and 50 um, regular in shape were obtained. These GVs effectively entrapped the fluorescent markers and appeared to be uni- or oligo-lamellar. When DDAB and PUCE, two cationic surfactant were used, a slightly larger number of GVs was obtained. When, on the other hand, oleate was used, no vesicles were found in the aqueous phase.
After these attempts, the experimental strategy was changed. Instead of using one lipid, mixtures of two lipids were employed. To form the emulsion and the interface we mixed a POPC solution (in mineral oil) in a 1:1 v/v ratio with another lipid solutions of the same concentration, in order to have 50% of negative charge (POPS, POPG and oleate) or positive (PUCE or DDAB). In all cases, significant increase in GVs production was observed.
Finally, we also tested the case when POPC only was used for the preparation of the w/o emulsion, whereas another (different) surfactant was used to create the macroscopic oil/water interface. Surprisingly, a greater number of GVs was obtained. It is clear that the charged lipid at the interface helps the formation of GVs, and that this condition is the optimal one for producing a large number of GVs.
In figure 11 it is reported the results of the case POPC/oleate. Whereas when POPC only was used the number of GVs per ml was around 20, the use of a POPC/oleate 1:1 mixture increased such value to ca. 150. If, however, oleate was used to form the interfase

whereas POPC was used for the emulsion, the GVs production yield was around 400/ml. Similar results have been obtained with other kinds of charged lipids (data not shown). In other words, separating two lipid species in the two phases (w/o emulsion and interface) brings about a significant increase of GVs production. Notice that this procedure possibly produces asymmetric membranes.

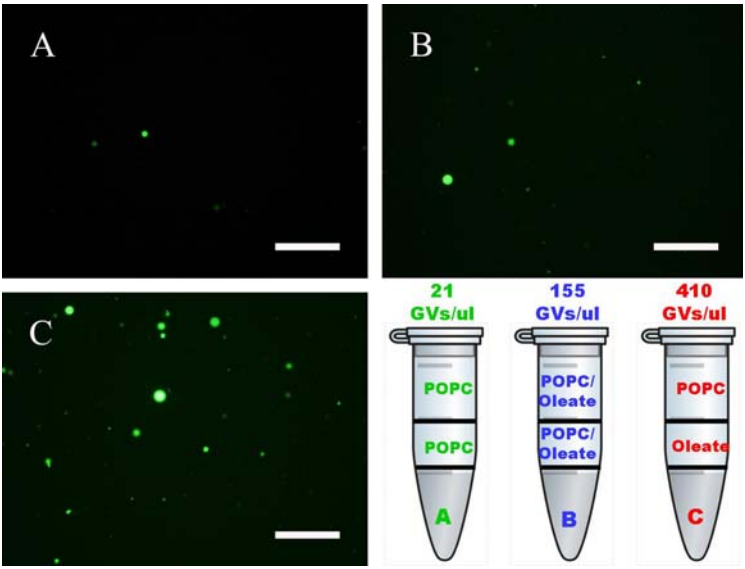


Fig. 11: Counts were performed using 10 photos of vesicles within the hemocytometer by the fluorescence microscopy, using the 10x magnification. For image analysis was used Image J software. A) GVs obtained with only POPC; B) GVs obtained with POPC and oleate mixed; C) GVs obtained with POPC and oleate unmixed. In the bottom right is shown the number per ul of GVs obtained and the phase patterns in the eppendorf in three cases. Size bar = 250um.

3.1.2. Effect of the outer aqueous volume

The next optimization step involved the exploration of other parameters such as the volume of the external buffer ("outer phase"), the volume of the internal buffer ("inner phase"), the volume of the w/o emulsion and the concentration of both aqueous buffers. All experiments were performed by using POPC-based w/o emulsion and one kind of charged lipids for the interface.

Lipid combinations used are as follows:

- POPC:POPG 1:1;
- POPC:POPS 1:1;
- POPC:oleate 1:1;
- POPC:DDAB 1:1;
- POPC:PUCE 1:1.

In the first series of experiments, we varied the volume of the external buffer, from 200 to 500 ul (10 mM Tris HCl/100 mM glucose/pH 7.5 was used). As the internal buffer, 10 mM Tris HCl/100 mM sucrose//50 uM calcein/pH 7.5 was used, and the volume of w/o emulsion as well as of the interphase was fixed to 150 ul.

Figure 12 and Table 3 summarize the result of this experiment, based on POPC:POPG lipid system.

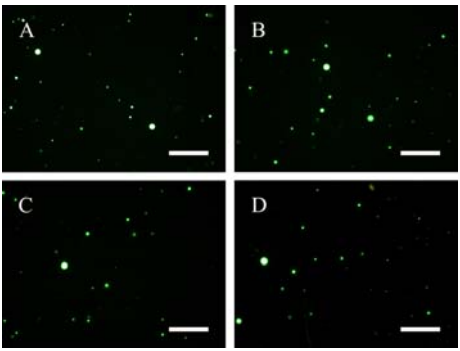


Fig. 12: Fluorescence images of POPC:POPG (1:1) GVs labeled with calcein. Outer buffer volume is in A) = 200ul; B) = 300ul; C) = 400ul and D) = 500ul. Size bar = 250um.

In addition to counting GVs, image analysis can be used for estimating other physical parameters like the average GVs' size, the average GVs' volume and consequently estimate the fraction of the volume – initially used to prepare the emulsion – that has been converted into GVs inner volume. For example, 30% entrapped volume means that the sum of all GVs' inner volume accounts for about 30% of the volume used to produce the w/o emulsion. The missing part can be ascribed to the amount of solution still solubilized in the oil even after the centrifugation (e.g. very small reverse micelles), or to the amount of solution, initially present as w/o droplets that instead of being converted into GVs has been released due to the competitive process of w/o droplet break at the oil/water interface (see below).

Tab. 3: Image analysis results about POPC:POPG GVs obtained with different outer buffer volume (200-500ul).

Vol. outer buffer (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
200	488	97600	9,62	1339	17,4
300	416	124800	8,08	848	14,1
400	304	121600	9,62	1852	30,0
500	258	129166	10,9	3619	62,3

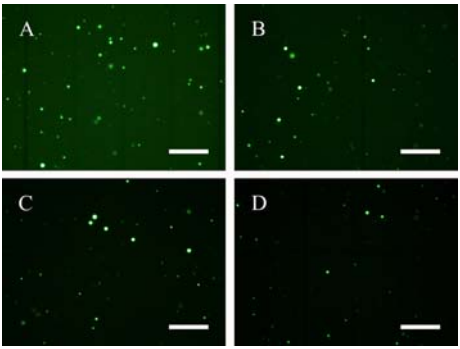


Fig. 13: Fluorescence images of POPC:POPS (1:1) GVs labeled with calcein. Outer buffer volume is in A) = 200ul; B) = 300ul; C) = 400ul and D) = 500ul. Size bar = 250um.

The quality of the four sample was quite good, as evident from the clean bright field, no fluorescence background and large GVs (> 40 um diameter). The number of GVs/mL reaches a steady value for outer volumes from 300 to 500 ul, but, due to size increase, the percentage of entrapped volume increases as the outer buffer volume. A similar approach has been carried out for other lipid. In particular, in Figure 13 and Table 4 results from the system POPC:POPS are reported. Here we can observe that the number of GVs/ml is similar to that one of the previous case, but their different sizes imply a minor entrapped volume.

Tab. 4: Image analysis results about POPC:POPS GVs obtained with different outer buffer volume (200-500ul).

Vol. outer buffer (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
200	614	122800	7,4	469,7	7,7
300	630	189000	6,8	434,1	11,0
400	614	245600	7,4	550,4	18,0
500	498	249000	7	349,5	11,6

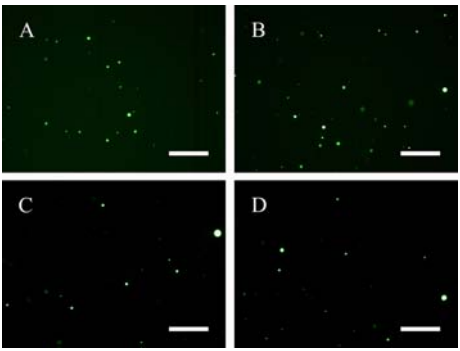


Fig. 11: Fluorescence images of POPC:oleate (1:1) GVs labeled with calcein. Outer buffer volume is in A) = 200ul; B) = 300ul; C) = 400ul and D) = 500ul. Size bar = 250um.

Moreover, when 200 ul outer buffer volume are used the background appears slightly green. Black backgrounds is instead present in the case of 400 and 500 ul, whereas the "300 ul" system has intermediate properties. The sample prepared with 400 ul outer volume appears to be the best.

The case of POPC:oleate is illustrated in Figure 14 and Table 5.

Tab. 5: Image analysis results about POPC:oleate GVs obtained with different outer buffer volume (200-500ul).

Vol. outer buffer (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
200	**380**	**76000**	**7,2**	**437,2**	**4,4**
300	**350**	**105000**	**9,1**	**862,2**	**12,1**
400	**188**	**75200**	**7,9**	**641,1**	**6,4**
500	**195**	**97500**	**9,8**	**1092,6**	**14,2**

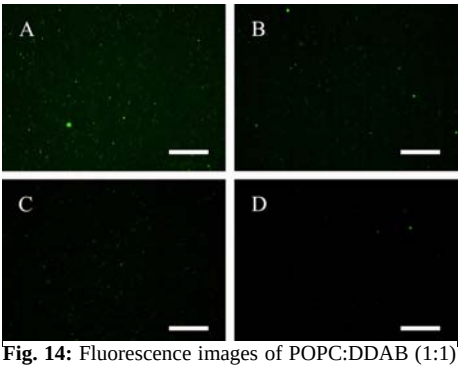


Fig. 14: Fluorescence images of POPC:DDAB (1:1) GVs labeled with calcein. Outer buffer volume is in A) = 200ul; B) = 300ul; C) = 400ul and D) = 500ul. Size bar = 250um.

As in the other two cases, POPC:oleate GVs are numerous and they are sized as those composed by POPC:POPS. The clean bright-field solution, and the very dark background (except for the case at 200 ul) show the good quality of the samples. The maximum number of GVs/ul is

found when 300 ul outer buffer are used, and decreases when larger volumes are used.
Moving to cationic lipids, the data referring to POPC:DDAB GVs are presented in Figure 15 and Table 6.
In this case, GVs are quite small but more numerous than when anionic lipids are used. The average size is around 4 um. The solution seems to be less clean than the previous samples (as evident by bright field images), and the fluorescent background is weakly green. The best conditions are found when the outer buffer volume is 300 ul. When 200 ul are used, the highly fluorescent background reveals that an higher amount of solutes has been released in the solution, whereas at higher outer buffer volumes (400 and in particular in the 500 ul), there is a drastic reduction in GVs number.

Tab. 6: Image analysis results about POPC:DDAB GVs obtained with different outer buffer volume (200-500ul).

Vol. outer buffer (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
200	2060	412000	4,3	75,3	4,1
300	1920	576000	4,2	82,6	6,3
400	870	348000	4,2	74,4	3,4
500	276	138000	4,3	93	1,7

Finally, in Figure 16 and Table 7 the system POPC: PUCE is analyzed.

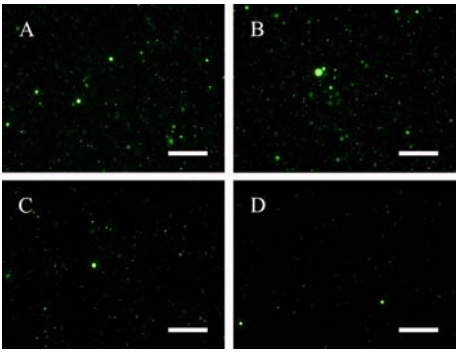


Fig. 16: Fluorescence images of POPC:PUCE (1:1) GVs labeled with calcein. Outer buffer volume is in A) = 200ul; B) = 300ul; C) = 400ul and D) = 500ul. Size bar = 250um.

Compared with the DDAB-containing GVs, PUCE-containing GVs are slightly larger, yet smaller than anionic GVs. In bright field the solution is clear, and the fluorescence background is completely black in all cases. Undoubtedly the best samples are formed when 200 or 300 ul outer volume are used, obtaining the greatest

number of GVs. In the other two cases, a reduced GVs number and size is observed.

Tab. 7: Image analysis results about POPC:PUCE GVs obtained with different outer buffer volume (200-500ul).

Vol. outer buffer (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
200	**5196**	**1039200**	**5,4**	**196,3**	**27,2**
300	**3692**	**1107750**	**5,5**	**204,1**	**30,1**
400	**2410**	**964000**	**5,2**	**170,1**	**21,9**
500	**1162**	**581000**	**4,9**	**132,7**	**10,3**

3.1.3. Effect of the w/o emulsion volume and of the interface

In the previous experiments, I have found that the best external buffer volumes are 300 and 400 ul, because by using 200 ul, some samples show a green background fluorescence, while when the volume is higher (500 ul), GVs are too diluted.

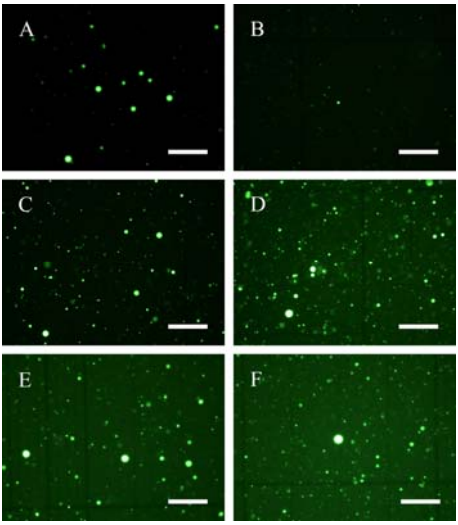


Fig. 17: Fluorescence images of POPC:POPG (1:1) GVs labeled with calcein. Emulsion volume is in A) = 100ul; B) = 200ul; C) = 300ul ;D) = 400ul; E) = 500ul and F) = 600ul. Size bar = 250um.

Thus, when assessing what is the best volume of emulsion, in the next series of experiments, the outer buffer volume was fixed to 300 ul. Different volumes of the w/o emulsion are now used (from 100 to 600 ul). The volume of the interface phase has also changed accordingly, by keeping constant to one the ratio between w/o emulsion and interface (i.e., the w/o emulsion and the interface volumes are equal).

Tab. 8: Image analysis results about POPC:POPG GVs obtained with different emulsion volume (100-600ul).

Vol. Emulsion (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
100	**1300**	**390000**	**8,8**	**1227**	**95,7**
200	**433**	**130000**	**5,0**	**161**	**2,1**
300	**1033**	**310000**	**14,9**	**5490**	**100**
400	**2425**	**727500**	**10,0**	**3090**	**100**
500	**1250**	**375000**	**15,0**	**6112**	**91,7**
600	**2300**	**690000**	**9,7**	**2127**	**48,9**

The kinds of lipids and aqueous buffers used are the same as in previous experiments.
In the Figure 17 and in Table 8 the analysis of POPC:POPG GVs is reported.
In the first set of samples is evident that by increasing the volume of emulsion, the green fluorescence background increases correspondingly. The background fluorescence is acceptable up to 300 ul of w/o emulsion, but when higher volumes are used, it is evident that solute has been largely released when GVs formed by crossing the interface. The percentage of entrapment volume is fair when w/o emulsion volume is between 300 and 500 ul, due to the larger vesicle size obtained in these systems.

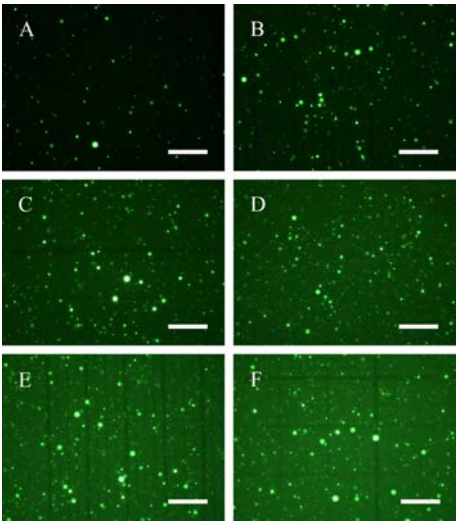


Fig. 18: Image analysis results about POPC:POPS GVs obtained with different emulsion volume (100-600ul).

The outcome of POPC: POPS GVs preparation is shown in Figure 18 and Table 10.

Tab. 10: Image analysis results about POPC:POPS GVs obtained with different emulsion volume (100-600ul).

Vol. Emulsion (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
100	**1775**	**532500**	**8,9**	**1149**	**122,4**
200	**2575**	**772500**	**8,6**	**1677**	**129,5**
300	**2675**	**802500**	**8,0**	**935**	**50,0**
400	**3375**	**1012500**	**7,2**	**894**	**40,7**
500	**5700**	**1710000**	**8,0**	**900**	**61,5**
600	**3350**	**1005000**	**8,6**	**1246**	**41,7**

At the lipid composition POPC:POPS, the increase of the emulsion volume brings about an increase of the fluorescence background already when the w/o emulsion volume is 300 ul. GVs in all samples have an average size between 8 and 9 um. Samples prepared from 100 or 200 ul emulsion have a high percentage of entrapment, whereas a lower percentage of entrapment is obtained when more than 300 ul emulsion was used, despite the high GVs number observed in these cases.

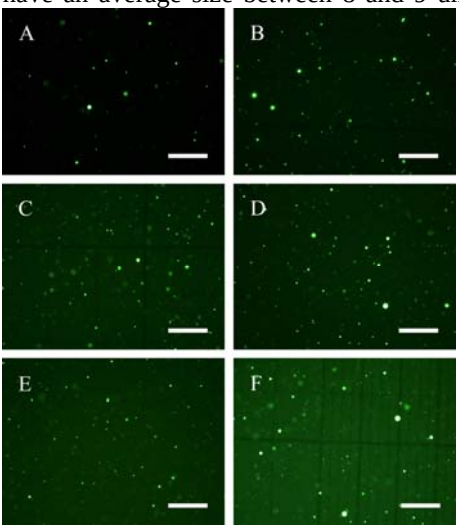


Fig. 19: Fluorescence images of POPC:oleate (1:1) GVs labeled with calcein. Emulsion volume is in A) = 100ul; B) = 200ul; C) = 300ul ;D) = 400ul; E) = 500ul and F) = 600ul. Size bar = 250um.

Next case is that one based on POPC:oleate GVs (see Figure 19 and Table 11).

In the case of POPC:oleate vesicles a similar pattern is observed. In particular an increase in background fluorescence is recorded starting from emulsion volume of 300 ul, even if less that when GVs are formed by other lipids. The number of GVs per ul, the total number and percentage of entrapment are also lower than what previously shown with other anionic phospholipid.

Tab. 11: Image analysis results about POPC:oleate GVs obtained with different emulsion volume (100-600ul).

Vol. Emulsion (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
100	**625**	**187500**	**10,0**	**1146**	**43**
200	**900**	**270000**	**7,6**	**786**	**21,2**
300	**1650**	**495000**	**7,7**	**550**	**18,1**
400	**1925**	**577500**	**7,7**	**830**	**24**
500	**2350**	**705000**	**5,6**	**239**	**6,7**
600	**1875**	**562500**	**6,4**	**629**	**11,8**

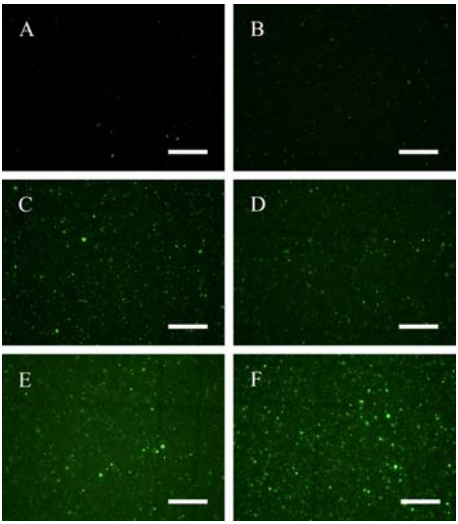


Fig. 20: Fluorescence images of POPC:DDAB (1:1) GVs labeled with calcein. Emulsion volume is in A) = 100ul; B) = 200ul; C) = 300ul ;D) = 400ul; E) = 500ul and F) = 600ul. Size bar = 250um.

As before, the same analysis has been carried out with cationic lipids. In Figure 20 and Table 12 the formation of GVs composed by POPC: DDAB is shown. As in the case of experiments on the variation of external buffer volume, the set of cationic lipid POPC:DDAB were characterized by a high GVs number, but having small size, namely between 5 and 7 um. The background appears dark up to 400 ul, but the percentage of entrapment is quite low, due to the small vesicle size.

Tab. 12: Image analysis results about POPC:DDAB GVs obtained with different emulsion volume (100-600ul).

Vol. Emulsion (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
100	**425**	**127500**	**7,1**	**345**	**8,8**
200	**833**	**250000**	**6,4**	**269**	**6,7**
300	**3050**	**915000**	**5,5**	**212**	**12,9**
400	**3300**	**990000**	**4,9**	**137**	**6,8**
500	**5175**	**1552500**	**5,6**	**330**	**20,5**
600	**5075**	**1522500**	**5,8**	**341**	**17,3**

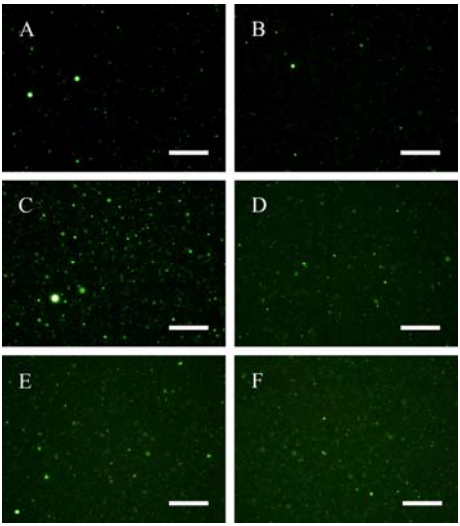


Fig. 21: Fluorescence images of POPC:PUCE (1:1) GVs labeled with calcein. Emulsion volume is in A) = 100ul; B) = 200ul; C) = 300ul ;D) = 400ul; E) = 500ul and F) = 600ul. Size bar = 250um.

Finally, GVs prepared from POPC:PUCE have been characterized as shown in Figure 21 and Table 13.

POPC:PUCE GVs behaves similarly than the POPC:DDAB ones. Small size and consequent low entrapment efficiency has been observed. The fluorescence background in the microimages becomes very evident only when 500 or 600 ul emulsion is used. The best sample can be considered that one obtained by starting from 300 ul w/o emulsion, which is comparable to what happens in the case of anionic lipids.

Tab. 13: Image analysis results about POPC:PUCE GVs obtained with different emulsion volume (100-600ul).

Vol. Emulsion (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
100	**1275**	**382500**	**6**	**297**	**22,7**
200	**1225**	**367500**	**4,4**	**63**	**2,3**
300	**2075**	**622500**	**6,1**	**482**	**20**
400	**2800**	**840000**	**4,4**	**102**	**4,3**
500	**2675**	**802500**	**4,5**	**109**	**3,8**
600	**2800**	**840000**	**5,3**	**387**	**10,9**

3.1.4. Effect of the inner buffer volume

In previous experiments we have verified that the best external buffer volumes are 300 and 400 ul, and that the best emulsion volume is 400 ul. Therefore we have chosen 400 ul both external buffer and emulsion. It now remains to estimate the best amount of aqueous buffer (internal buffer) for the preparation of the emulsion. The internal volume has been explored in the range: 1 (0,25% v/v), 4 (1%), 8 (2%) and 16ul (4%), by using 400 ul of oil phase. Aqueous buffers and lipids are the same as the other two series of experiments.

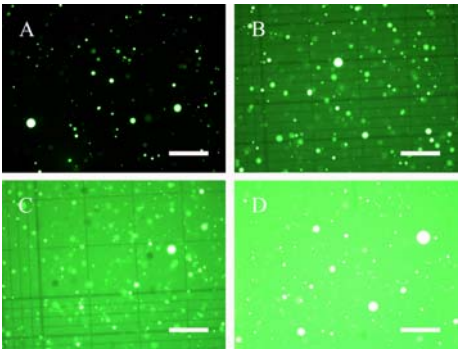


Fig. 22: Fluorescence images of POPC:POPG (1:1) GVs labeled with calcein. Inner buffer volume is in A) = 1ul; B) = 4ul; C) = 8 and D) = 16ul. Size bar = 250um.

The first system to be explored is the anionic GVs composed by POPC:POPG (Figure 22, Table 14).

Tab. 14: Image analysis results about POPC:POPG GVs obtained with different inner buffer volume (1-16ul).

Vol. inner buffer (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
1	1525	610000	10,4	2525	100
4	3475	1390000	10,7	2864	99,5
8	6450	2580000	11	2479	80
16	10150	4060000	8,5	1592	40,4

From images acquired by fluorescence microscopy, it is clear that increasing the volume of the internal buffer brings about the increase of the fluorescence background. Only when a w/o emulsion created with 0.25% of aqueous phase is transformed into vesicles, the background results dark. This means that when higher aqueous volumes are emulsified, more w/o droplets are formed, but consequently more of them are broken at the macroscopic oil/water interface, so that the fluorescent solute is released in the lower aqueous phase. But, interestingly, the increase of the volume of internal buffer leads to a significant increase of the GVs number. It is therefore clear that the best conditions for GVs production should be a compromise between high numerical density and solute release. The number of GVs per ul, the total GVs number and the average entrapment percentage are good in all four samples, and the average size is always around 10 um.

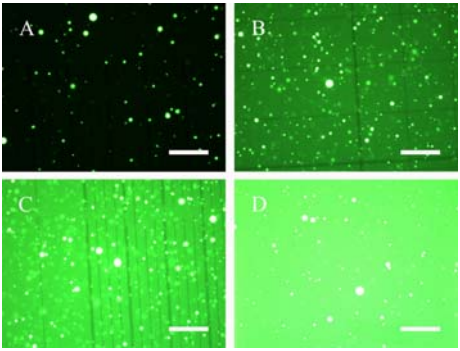


Fig. 23: Fluorescence images of POPC:POPS (1:1) GVs labeled with calcein. Inner buffer volume is in A) = 1ul; B) = 4ul; C) = 8 and D) = 16ul. Size bar = 250um.

Very similar results have been obtained by working with the POPC:POPS GVs system (Figure 23, Table 15).

Tab. 15: Image analysis results about POPC:POPS GVs obtained with different inner buffer volume (1-16ul).

Vol. inner buffer (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
1	1425	570000	11,9	2082	100
4	4650	1860000	8,7	1128	52,4
8	7825	3130000	8,9	906	35,5
16	9175	3670000	8,1	804	18,4

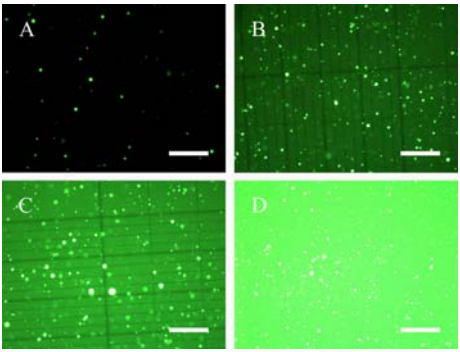


Fig. 24: Fluorescence images of POPC:oleate (1:1) GVs labeled with calcein. Inner buffer volume is in A) = 1ul; B) = 4ul; C) = 8 and D) = 16ul. Size bar = 250um.

Interestingly, the good results obtained with anionic phospholipid-based GVs have been also found in anionic POPC:oleate GVs (Figure 24, Table 16). All the samples contained GVs with average size between 8 and 11 um, and there is a good percentage of entrapment. In this series of experiments too, it is evident that as the volume of the internal buffer increases, a parallel increasing the number of w/o droplet can be observed, and consequently an increase of the "breakage" event at the oil/water interface, leading to high fluorescent background.

Tab. 16: Image analysis results about POPC:oleate GVs obtained with different inner buffer volume (1-16ul).

Vol. inner buffer (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
1	725	290000	11,6	1331	38,6
4	3340	1336000	8	509	17
8	4825	1930000	8,5	988	23,8
16	10150	4060000	7,5	514	15,2

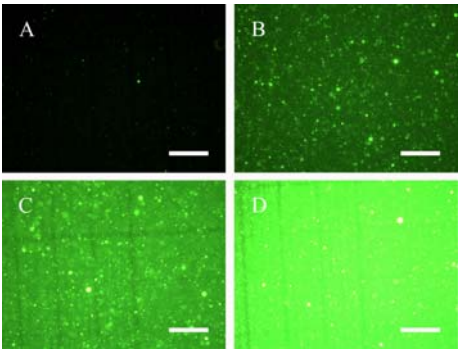


Fig. 25: Fluorescence images of POPC:DDAB (1:1) GVs labeled with calcein. Inner buffer volume is in A) = 1ul; B) = 4ul; C) = 8 and D) = 16ul. Size bar = 250um.

The cationic systems investigated are, as always, based on POPC:DDAB and POPC:PUCE. In Figure 25 and Table 17 micrographs and image analyis of the POPC:DDAB GVs are reported.

Tab. 17: Image analysis results about POPC:DDAB GVs obtained with different inner buffer volume (1-16ul).

Vol. inner buffer (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
1	4275	1710000	4,4	89	15,3
4	5440	2176000	5,8	308	16,8
8	8600	3440000	6,1	367	15,8
16	6625	2650000	6,4	582	9,6

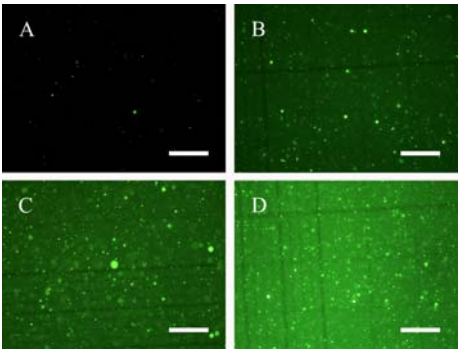


Fig. 26: Fluorescence images of POPC:PUCE (1:1) GVs labeled with calcein. Inner buffer volume is in A) = 1ul; B) = 4ul; C) = 8 and D) = 16ul. Size bar = 250um.

When POPC:DDAB GVs are prepared by using different inner buffer volume, it is observed that by increasing the volume of the internal buffer more vesicles are produced, but at the same time the background become more fluorescent. This is due to release of fluorescent content during the GV

formation by crossing the oil/water interface. The percentage of entrapment is low as the average size of vesicles that is between 4 and 6 um.
Finally, the analysis of POPC:PUCE GVs is shown in Figure 26 and Table 18.
The general pattern observed for all lipid system is also found in POPC:PUCE GVs. The only difference is that there is a lower green background intensity in the fluorescent images. The average size is between 6 and 7 um and it is intermediate between those of anionic lipid vesicles and those obtained with POPC:DDAB.

Tab. 18: Image analysis results about POPC:PUCE GVs obtained with different inner buffer volume (1-16ul).

Vol. inner buffer (ul)	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
1	1100	440000	7,3	580	25,5
4	2900	1160000	7	448	13
8	2900	1160000	5,8	252	3,7
16	10150	4060000	5,8	320	2,7

3.1.5. Summary of the first three optimization steps
The result of these first three series of optimization experiments showed a common trend in all lipid sets used. When the external buffer volume has been varied, the best results were obtained with the larger volumes. Although the number of vesicles per ul is greater when smaller volumes were used, the *total* number of GVs obtained is always greater with larger volumes. It is evident that larger internal volume gives more w/o droplets, and therefore more GVs. But as observed in several experiments, this also increment the amount of released solute in the final aqueous phase, due to droplet breakage when crossing the oil/water interface. Higher oil volumes also increment the number of GVs, probably because the resulting w/o emulsion is more stable, so that w/o droplets do not coalesce, and their number does not decrease.
In conclusion, using larger volumes of internal, external buffer and emulsion, we obtain a greater total number of GVs. The drawback is that these parameter also lead to solute release in the aqueous phase. However, since GVs can be easily centrifuged the external phase can be exchanged by resuspending the GVs pellet in a desired volume of fresh buffer (this also allow to concentrate GVs).

Therefore, we have chosen the following “standard” condition to prepare GVs: (1) 500 ul external buffer, (2) 500 ul mineral oil interface phase, containing lipids, (3) 500 ul mineral oil emulsion phase, containing lipids, (4) variable amount of internal solution (0.25-0.5% for low release, 2 to 4% for high GVs number).

3.1.6. Effect of the buffer concentration of the internal and external aqueous phase

Maximized the number of GVs produced by the emulsion inversion method, it was then evaluated the effect of the concentration of Tris HCl buffer. Therefore, using the same lipid mixtures, it was assessed the effect of the buffer concentration (from 5 to 200 mM). Notice that the other buffer components (100 mM sucrose in internal buffer and 100 mM glucose in external buffer have been not changed).

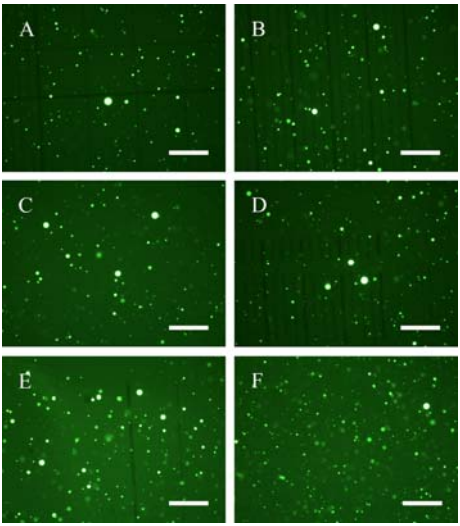


Fig. 27: Fluorescence images of POPC:POPG (1:1) GVs labeled with calcein. Inner and outer buffer Molarity is in A) = 5mM; B) = 10mM; C) = 20mM ;D) = 50mM; E) = 100mM and F) = 200mM. Size bar = 250um.

The first system investigated was POPC:POPG GVs (Figure 27 and Table 19).

Tab. 19: Image analysis results about POPC:POPG GVs obtained with different inner and outer buffer Molarity (5-200mM).

Inner and outer buffer mM	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
5	**1720**	**688000**	**11**	**4155**	**71,5**
10	**2025**	**810000**	**9,6**	**1024**	**20,7**
20	**2025**	**810000**	**10,1**	**1674**	**33,9**
50	**1700**	**680000**	**6,6**	**470**	**8**
100	**2075**	**830000**	**8,1**	**638**	**13,2**
200	**2950**	**1180000**	**7,4**	**601**	**17,7**

At low buffer concentration (5-20 mM), and there is also a good percentage of solute entrapment. By increasing the buffer concentration, smaller GVs are produced, so that the entrapment percentage is also lower. In Figure 28 and Table 20 it is shown the characterization of POPC:POPS GVs.

In the case of POPC:POPS mixture, the buffer concentration did not affect significantly the number of GVs. The best sample was obtained at a buffer concentration of 20 mM.

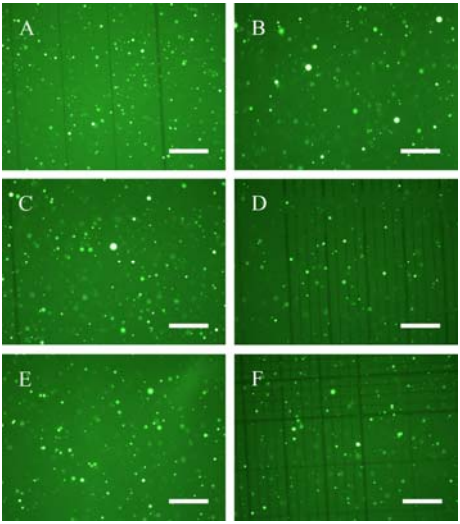


Fig. 28: Fluorescence images of POPC:POPS (1:1) GVs labeled with calcein. Inner and outer buffer Molarity is in A) = 5mM; B) = 10mM; C) = 20mM ;D) = 50mM; E) = 100mM and F) = 200mM. Size bar = 250um.

Tab. 20: Image analysis results about POPC:POPS GVs obtained with different inner and outer buffer Molarity (5-200mM).

Inner and outer buffer mM	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
5	2800	1120000	7,8	492	13,8
10	2300	920000	9,3	910	21
20	4750	1900000	8,6	940	44,6
50	1900	760000	8,2	1040	19,8
100	2775	1100000	8,1	603	16,7
200	1975	790000	8	609	12

As final anionic lipid system, the characterization of POPC:oleate GVs is shown in Figure 29 and Table 21.

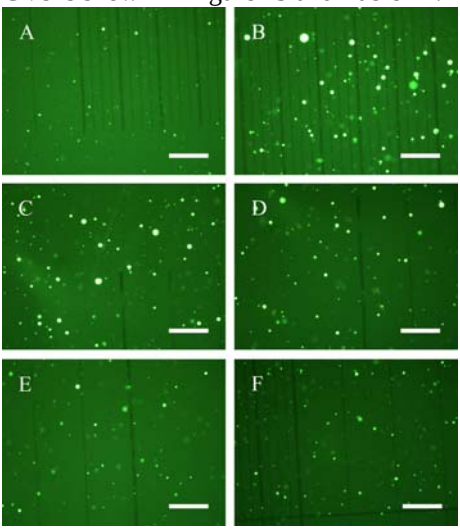


Fig. 29: Fluorescence images of POPC:oleate (1:1) GVs labeled with calcein. Inner and outer buffer Molarity is in A) = 5mM; B) = 10mM; C) = 20mM ;D) = 50mM; E) = 100mM and F) = 200mM. Size bar = 250um.

In this series of experiments the best results were obtained at a buffer concentration between 10 and 50 mM. In these samples was observed a considerable number of GVs, with an average size around 10 um and with a good percentage of entrapment. In the 5 mM sample, even if a large number of GVs is formed, their average size is smaller, ca. 6 um. While in 100 and 200 mM samples there is a lower GVs yield.

Tab. 21: Image analysis results about POPC:oleate GVs obtained with different inner and outer buffer Molarity (5-200mM).

Inner and outer buffer mM	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
5	1875	750000	6	247	46,4
10	2100	840000	10,7	1944	40,8
20	1975	790000	9,4	1111	21
50	1500	600000	9,7	1146	17,2
100	900	360000	9	1060	9,5
200	1200	480000	7,5	567	6,8

The cases of cationic GVs have been illustrated in Figure 30 and Table 22.

Maximum production of GVs is observed at a concentration of 20 mM but also in 10 and 50 mM samples there is a high yield. This behavior is similar to the POPC:oleate case. At high buffer

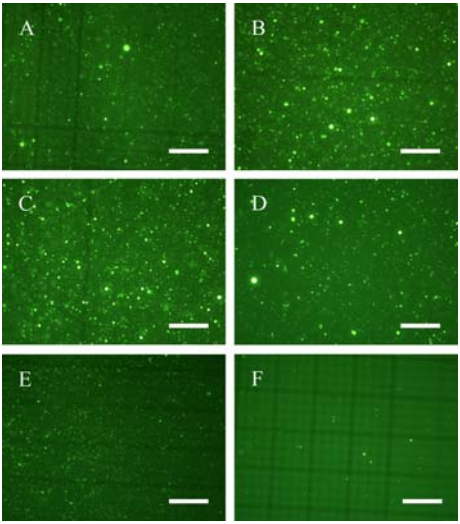


Fig. 30: Fluorescence images of POPC:DDAB (1:1) GVs labeled with calcein. Inner and outer buffer Molarity is in A) = 5mM; B) = 10mM; C) = 20mM ;D) = 50mM; E) = 100mM and F) = 200mM. Size bar = 250um.

concentration, the production of GVs is somehow reduced, in terms of size and number (and therefore entrapment yield). Finally, in Figure 31 and Table 23 the results about the POPC:PUCE GVs are shown.

For the POPC:PUCE lipid mixture, the result is very similar to that obtained with POPC: DDAB, although in general in all the samples there is a much smaller number of GVs than the previous case.

Tab. 22: Image analysis results about POPC:DDAB GVs obtained with different inner and outer buffer Molarity (5-200mM).

Inner and outer buffer mM	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
5	**2875**	**1150000**	**7,3**	**552**	**15,9**
10	**13625**	**5450000**	**5,8**	**243**	**33,1**
20	**21575**	**8630000**	**5,5**	**201**	**43,5**
50	**11075**	**4430000**	**5,7**	**220**	**24,4**
100	**1750**	**700000**	**4,7**	**151**	**2,6**
200	**1375**	**550000**	**4,8**	**96**	**1,3**

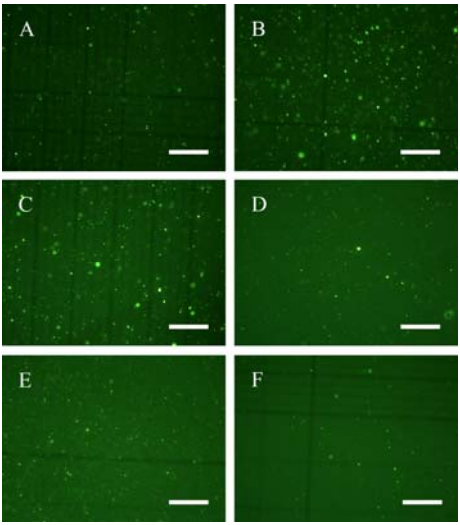


Fig. 31: Fluorescence images of POPC:PUCE (1:1) GVs labeled with calcein. Inner and outer buffer Molarity is in A) = 5mM; B) = 10mM; C) = 20mM ;D) = 50mM; E) = 100mM and F) = 200mM. Size bar = 250um.

This series of experiments have clarified the effect of the buffer concentration on the formation of GVs in the emulsion inversion method. Although in all concentrations and in all lipid mixtures the formation of GVs was observed, these systems a different GVs number and size has been found in all cases. This is ultimately due to the fine balance between hydrophobic, hydration and electrostatic forces that determine the formation and the stability of w/o emulsion droplets and later GVs. Phospholipid mixtures as POPC:POPG and POPC:POPS behave similarly, whereas other kind of surfactants like oleate, DDAB, and PUCE have a different behavior, and can be considered as a separate group. It is interesting that anionic vesicles formed by phospholipids (POPG or POPS) have different features than those formed by fatty acids (oleate).

Tab. 23: Image analysis results about POPC:PUCE GVs obtained with different inner and outer buffer Molarity (5-200mM).

Inner and outer buffer mM	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
5	**1825**	**730000**	**5,1**	**193**	**3,5**
10	**2925**	**1170000**	**5,9**	**289**	**8,5**
20	**3600**	**1440000**	**6,2**	**328**	**11,8**
50	**1000**	**400000**	**5,1**	**178**	**1,8**
100	**975**	**390000**	**6,2**	**288**	**2,8**
200	**650**	**260000**	**5,9**	**194**	**1,3**

3.1.7. Further investigation on the POPC:oleate system

In order to understand why charged lipids at the oil/water interface are so important to get an high GVs yield, we have designed an experiment where different mixture POPC:oleate were used, instead of pure oleate or pure POPC interface. We have chosen oleate due to the fact that this molecule is highly mobile (it is the only single-chain surfactant among the group investigated) and can quickly cover the oil/water interface. In fact, in one of the original Weitz paper (Pautot *et al*., 2003a) there has been reported that the working hypothesis for the effectiveness of GVs formation is related to the physical extension of the surfactant monolayer at the oil/water interface and to its rate of reconstitution. We reasoned that by putting a charged, highly mobile surfactant, we facilitate the interface monolayer assembly, and therefore obtain more GVs.

Eleven samples were prepared using a w/o emulsion from POPC and an interface phase containing POPC and oleate at different ratios (100:0, 90:10, etc.).

As for the optimization experiments, for each sample GVs were collected and characterized by means of direct counting (hemocytometer) and fluorescence microscopy (Figure 32 and Table 24, Graph 1). Images were captured by confocal microscopy and analyzed by Image J software (Abramoff *et al*.,2004).

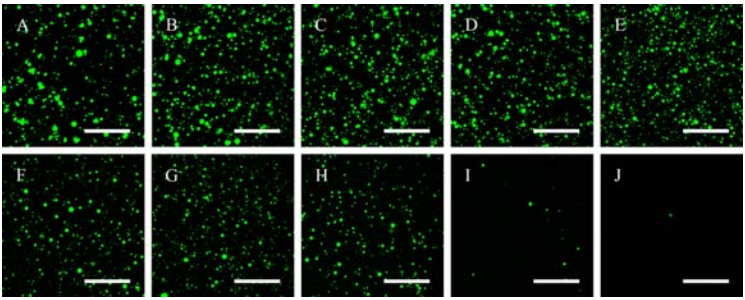
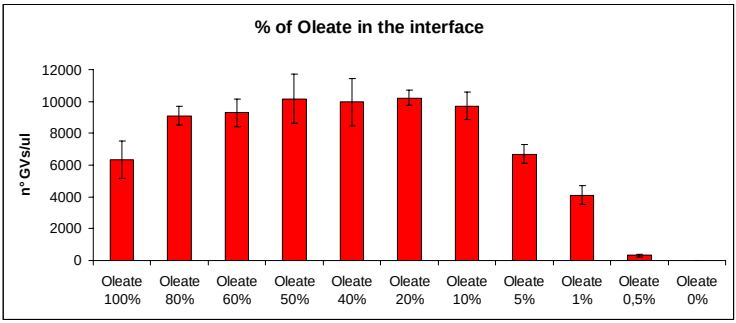


Fig. 32: Confocal images of POPC:oleate (1:1) GVs labeled with FITC-Dex. The percentage of oleate at the interface is A) = 100%; B) = 80%; C) = 60%; D) = 40%; E) = 20%; F) = 10%; G) = 5%; H) = 1%; I) = 0.5% and J) = 0%. Size bar = 250um.

Tab. 24: Image analysis results about POPC:oleate GVs obtained with different percentage of oleate at the interface (100-0%).

% of Oleate in the interface.	Nº GVs/ul	Nº GVs total	size (um)	vol. entrapped (um^3)	% vol. entrapped
100%	6325	1265000	10	1588	20
80%	9100	1820000	9	1128	21
60%	9298	1859667	9	1009	19
50%	10175	2035000	7	568	12
40%	9968	1993667	8	778	16
20%	10233	2046667	7	513	11
10%	9717	1943333	7	379	7
5%	6692	1338333	7	512	7
1%	4120	824000	9	628	5
0,5%	327	65333	8	511	0
0%	20	4000	7	655	0

Graph 1: Number of POPC:oleate GVs per ul obtained with different percentage of oleate at the interface (100-0%).



It is evident that the presence of oleate (even at low amount, i.e. remarkably up to 0.5%) at the interface is essential for GVs production. In fact, as already observed in optimization experiments when POPC only was placed at the interface, essentially no GVs are produced. This can be explained by the inability of POPC of quickly recovering the macroscopic interface after few w/o emulsion have crossed it and destroyed its ordered structure. In contrary, oleate might quickly reassemble in such a way that the interface monolayer is constantly replaced. Notice that if we consider a macroscopic interface of about 1 cm^2, it requires about 10^{14} oleate molecule for a complete monolayer. Since the interface phase contains ca. 10^{17} lipid

molecules (500 ul, 1 mM), 10^{15} of which are oleate (when oleate 0.5% is used), it means that there at least 10 times more oleate to form a complete interface. The maximum production of GVs is observed when the amount of oleate is between 20 and 50%.

3.1.8. Direct observation of GVs formation

We speculate that it would be very informative to visualize the process of GVs formation by direct imaging the interface during the droplet transfer (from oil to water). In order to observe a different behavior, we studied the case of pure POPC interface and POPC:oleate 1:1 interface.

At this aim, 200 ul of buffer outside were put inside a microscope slide well (IBIDI), and it was carefully stratified above it 200 ul of the interface phase.. Then 200 ul of a calcein containing w/o emulsion were added above the previous two layers. The interface was followed by real-time video-microscopy for bright field and fluorescence imaging.

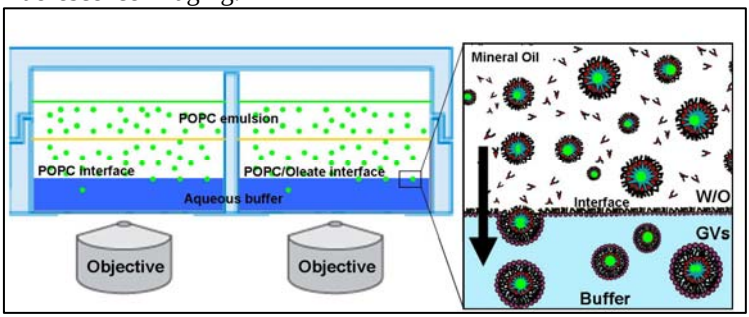


Fig. 33: Scheme of the emulsion inversion method within the µ-Slide 8 well (Ibidi). Above the aqueous buffer solution was layered a lipid solution in mineral oil to create the oil-water interface. In the first case was added only POPC while in the second POPC:oleate in a ratio 1:1. Subsequently, in both cases an emulsion made with only POPC containing FITC-Dex was added. The experiment was observed under the fluorescent and bright field microscope in real-time. In the right panel there is a pattern about what happens at the interface.

Figures 34 and 35 show images captured from the respective videos. Firstly, we observed the interface before the addition of the w/o emulsion. In the pure POPC interface, the spontaneous formation of numerous large aggregates of large vesicles or o/w droplets (up to 250 um) is observed. These aggregates grow in time and remain attached at the interface. In contrary, in the case of POPC:oleate

interface, only a very small number of (small, < 50 um) aggregate was found. This means that a qualitatively superior (smooth) interface is formed when oleate is present, probably due to its higher mobility that allows the establishment of quasi-equilibrium state, i.e., approaching the most stable one. POPC interface with its irregularities is reminiscent of the classical behavior of POPC in aqueous system, often characterized by "frozen" states, i.e., structures that form spontaneously without exploring different states due to kinetic reasons.

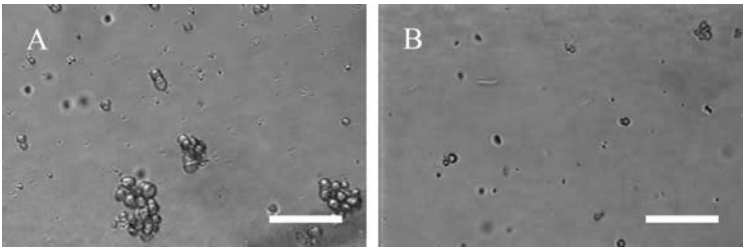


Fig. 34: Bright field images captured at the oil-water interface before adding emulsion. A) = POPC alone and B) = POPC:oleate mixed 1:1. Size bar = 250um.

Next, we directly visualize the interface after w/o emulsion addition on the top of the interface bilayer, in order to observe the formation of GVs (Figure 35).

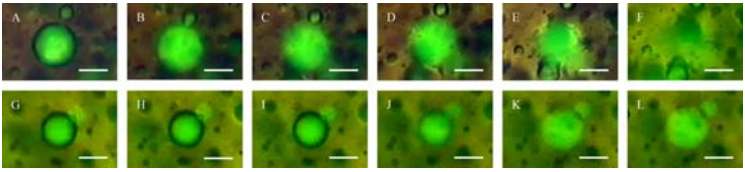


Fig. 35: Water in oil droplets as they pass through the oil-water interface. When the interface is composed of only POPC, the droplet through the interface, becomes a GVs but then bursts (A-F) while with POPC:oleate after crossing the interface, the GVs that has formed is stable (G-L). Images from A) to F) and from G) to H) were acquired every 500ms. Size bar = 10um.

In the case of POPC interface (Figure 35, panels A to F), the majority of droplets, after approaching the interface, burst immediately after crossing the interface, as evidenced by the release of calcein in the aqueous phase (i.e. the diffuse green fluorescent cloud in panel D to F). This behavior can be ascribed to imperfect monolayer interface formed by POPC, especially after interface

depletion/disruption after the passage of first droplets. In contrary, in the case of POPC:oleate interface (Figure 35, panels G to L), the majority of the droplets are able to cross the interface, aquire the outer lipid leaflet, become GVs, and keep their content. This can be explained by a rapid equilibrium between micellar or monomeric oleate, dissolved in oil, and the macroscopic oil/water interface. The resulting vesicles were often found hanging on the interface, as reported by Homma (Ohno et al., 2009), but in other case they spontaneously move from the interface to the outer buffer, driven by the density difference (sucrose inside, glucose outside). We conclude that centrifugation is simply a mean of effectively produce GVs in higher yields due to constant passage of w/o droplets through the interface, which is presumably continuously fed by new oleate.

3.2 Relevance of the emulsion inversion (droplet transfer) method and its optimization for studying cell models

The "emulsion inversion" formation method, as described by several authors (see the Introduction), it is a very advantageous to produce uni- or oligo-lamellar GVs in large quantities, capable of entrapping water soluble solutes in their inner space without excessive dispersion. Therefore, this method is very useful for the study of models of early cells, because it can produce functionalized GVs, with an asymmetric membrane and different combinations of lipids. Important solutes as DNA, RNA, proteins, enzymes, cofactors, nutrients, etc.. can be easily entrapped, whereas this step is critical in other preparation methods. Moreover these GVs can be easily manipulated so that the external environment can be changed at will. It is also foreseen that the combination of GVs and microfluidic devices might further improve the technology of minimal cells.
The drawback of this method, on the other hand, was known from the very beginning, consisting in its poor reproducibility, low GVs yield and small percentage of entrapment of water soluble solutes. In the first part of my work, therefore the several factors affecting the performance of the methods have been studied in detail. As a result, we have outlined an experimental protocol that describe the highly reproducible production of GVs at high yield (10000 GVs/ul), that can be further diluted or concentrated depending on the purpose of the experiment. It was possible to obtain GVs with an internal environment different from that outside, and it was possible to entrap

a wide range of water-soluble solutes as calcein (a small soluble molecule), FITC and RITC-dextran (sugars), allophycocyanin (APC), phycoerythrin (PE) (proteins) in their aqueous core. Preliminary results have extended this method to the entrapment of nucleic acids and enzymes (data not shown).

In previous paragraph we have reported a detailed analysis of anionic and cationic GVs. It is in fact important, from the experimental viewpoint, being able to prepare such oppositely charged vesicles for carrying out experiment of GVs interactions, driven by electrostatic forces. One of the main issue in the formation of early cells, actually is related to the possibility of GVs fusion in order to mix their content. There are indeed some reports on the study of vesicle fusion as a model of early cell interaction (Caschera *et al*., 2010; Sunami *et al*., 2010). Preliminary experiments, carried out during my PhD work have shown that GVs fusion is not at all an easy process. In fact, by mixing oppositely charged vesicles (e.g. POPC:POPG versus POPC:DDAB), each containing a different fluorescent dye, it was not possible to observe the spontaneous fusion event in the conditions used. Further investigations are in progress in order to understand the reasons for such low reactivity, that markedly differs from the case of conventional (submicrometric) vesicles (Caschera *et al*., 2010). It should be noticed, however, that previous reports from the group of McDonald (Pantazatos and MacDonald, 1999) reported the GVs fusion only after electric manipulation of GVs which were places almost in contact before the fusion event could occur. We aim to use cationic and anionic GVs as fusogenic vesicles for a stepwise construction of minimal cell model (each vesicle brings some of the ingredients required for reaching a minimal molecular complexity to carry out biomolecular reactions, such as protein synthesis, or a metabolic route).

The relatively low reactivity of GVs with respect to fusion suggests that the interaction between GVs occurs more effectively when these particles are in close contact. We have speculated about a possible scenario that realistically simulate primitive systems and investigated it experimentally. It turned out that an important aspect of vesicle chemistry was simply ignored in previous research, i.e. the issue of vesicle association and cooperation, or – stated in different way – the importance of collective patterns versus the focus on the dynamics of individual compartments. This will be the topic of the second part of the work.

3.3 GVs colonies as a model of primitive cell colonies: formation and characterization.

In large part of the literature on the origin of early life on Earth, it has been often discussed the emergence of living units from separated components, always looking at the event as a transition from unorganized mixtures of molecules to a spatially and functionally organized molecular *system*, enclosed by a semi-permeable membrane. However, this approach looks at the problem from a perspective that we can define "individual". Here we mean that not enough attention has been devoted to the role of interactions among cells, at their cooperation, and synergic effects.
Looking for example at the problem of molecular entrapment, it is difficult to imagine how several different components could have been entrapped in the same "individual" cells. A symbiogenic development of cellular life, where association of cells can fuse and exchange solutes each other, could lead to more complex behaviors and encourage the development of a capacity for life. We guess that it is useful to investigate in more detail, instead, whether and at what extent possible cooperation between cells could have helped in the primitive context, not only from the viewpoint of solute entrapment, but also providing some physical or evolutive advantage for *communities of cell-like systems* as opposite to individual compartments.
Therefore, we decided to study the association and the fusion of GVs as a model for symbiogenesis, where a cooperative mechanism could lead to a higher level of complexity and help in developing novel functionalities that do not exist in the single vesicles.
As noticed before, GVs fusion is not so easy as expected. We looked for new strategies for GVs fusion, mediated by preventive strong interaction with each other, bringing them the one close the other, in order to obtain a close association. We defined such vesicle aggregates as GVs colonies, in order to emphasize the close parallelism between in vitro and in vivo systems, even if at this stage such analogy is necessarily partial.
In order to create a model of primitive colonies we exploited the electrostatic attraction, a very basic and long-range physical force that had to be present also in ancient time. Since fatty acid vesicles are the best candidates for being primitive cell model (Deamer and Dworkin, 2005) we reasoned that in the plausible presence of a

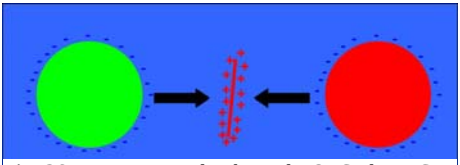


Fig. 36: Two negatively charged POPC:oleate GVs are attracted each other thanks to positively charged PLA bridge.

positively charged peptide, the aggregation should occur. As model cationic peptide we employed poly(arginine) (PLA; MW = 7.5 kDa). It is important to notice that the emergence of homooligomers of amino acids is considered plausible in prebiotic conditions, thank to the well known *N*-carboxyanhydride condensation reaction (Barlett, and Jones, 1957).

From the practical viewpoint, we mixed negatively charged GVs (POPC:oleate 1:1) with PLA. It is expected that due to their opposite charge, GVs and PLA form complex of the form $(GVs)_n(PLA)_m$. A simplified scheme is illustrated in Figure 36.

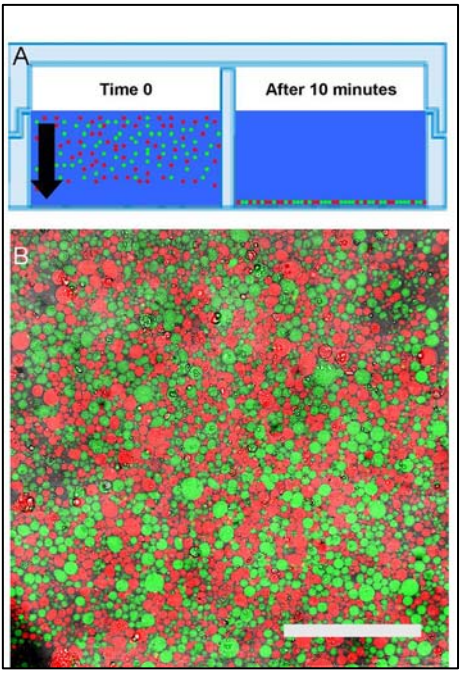


Fig. 37: GVs sedimentation within the µ-Slide 8 well (Ibidi). All GVs that are placed inside the well, they settle on the bottom after about 10 minutes (A). Confocal image of two population of POPC:oleate GVs on the bottom of the microscopic slide well (B). Size bar = 250um.

In order to visualize the different GVs composing the clusters, we have prepared and mixed in a slide well (IBIDI) two different GVs population by using two different fluorescent water-soluble markers, namely FITC-dextran (green fluorescent marker) and RITC-dextran (red fluorescent marker).

After a few minutes, thanks to the higher density of GVs when compared with the external buffer (100 mM sucrose versus 100 mM glucose) the GVs are found on the bottom of the well, forming

GVs layer, whose density depends on the concentration of GVs (Figure 37).

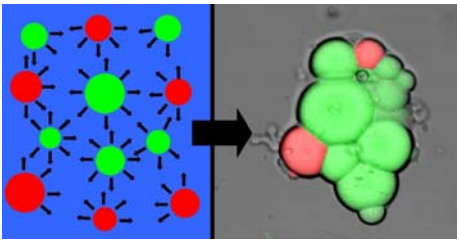


Fig. 38 : GVs colonies formation pattern. After the addition of PLA, the GVs begin to attract (A) and to attack (B) each other forming a colony. In B) there is a confocal image of a POPC:oleate GVs colony which consists of GVs labeled with FITC or RITC-Dex. Size bar = 10um.

To the so immobilized GVs, PLA is added as aqueous solution. Immediately after addition of PLA, GVs move towards each other, stick together and form colonies of various size. In the first seconds, the process is very fast, and keeps on for about half an hour. When the final state is reached, GVs colonies are easily distinguished as immobilized object lying on the surface of the well (Figure 38)

3.3.1 Effect of PLA and GVs concentration

The process of the formation of colonies has been characterized by changing the variables affecting its occurrence.

The first step was to test the threshold concentration of PLA. Two different populations of fluorescent GVs were produced and mixed (POPC:oleate marked FITC-Dextran, and POPC:oleate GVs marked RITC-Dextran). In the first well water was added as negative control,

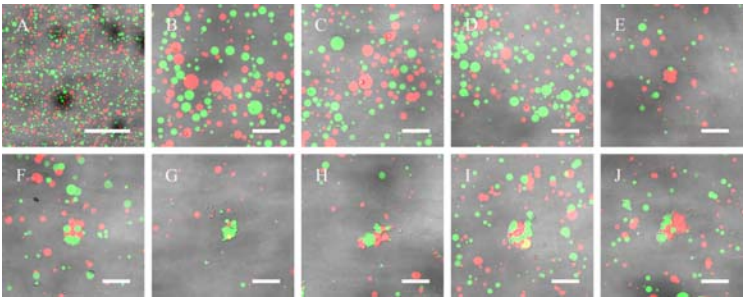


Fig. 39: GVs colonies formation from two population of POPC:oleate GVs labeled FITC or RITC-Dex at different PLA concentration. Confocal image A) shows the initial situation before the addition of PLA; size bar = 250um. Image B) shows the negative control at the final point. In C) there is the image obtained at the final point with a PLA concentration equal to 0.5ug/ml. In D), the PLA is 1ug/ml; in E) 2.5; in F) 5; in G) 10; in H) 25, in I) 50 and in J) 100ug/ml. Size bar (from B) to J)) = 50um.

confirming that no colonies are formed. PLA was then added to each separate sample at increasing concentrations (see Figure 39), from 0.5ug/ml to 200ug/ml.
For each sample, the dynamics of GVs colonies formation was followed in time via confocal microscopy, operating in time-lapse mode (incubation time: 30 min). Representative images are shown in Figure 39. Panel A shows the initial state before the addition of PLA. Panel B shows the negative control (water addition) at the final point. The other images refer to the final state at different PLA concentration, from 0.5 to 100 ug/ml. It is evident that GVs colonies form only when PLA is added above the threshold concentration of 2.5 ug/mL. Above the PLA concentration of 100 ug/ml, GVs become unstable and burst.
The second step was to verify whether the formation of colonies depended on the number of GVs initially present in the slide well. As in the previous experiment, POPC:oleate GVs, containing FITC-dextrane or RITC-dextrane were prepared and their concentration evaluated as shown before, and adjusted at the value of 10000 GVs/ul. After 1:1 mixing, the GVs were diluted with fresh buffer in order to obtain 7 samples (from 10000 to 500 GVs/ul). After sedimentation in the slide well, an equal amount of PLA (2.5 ug/ml) was added as before and the behavior registered by confocal microscopy time-lapse imaging (Figure 40).
Colonies are always observed. The only difference lies in the number

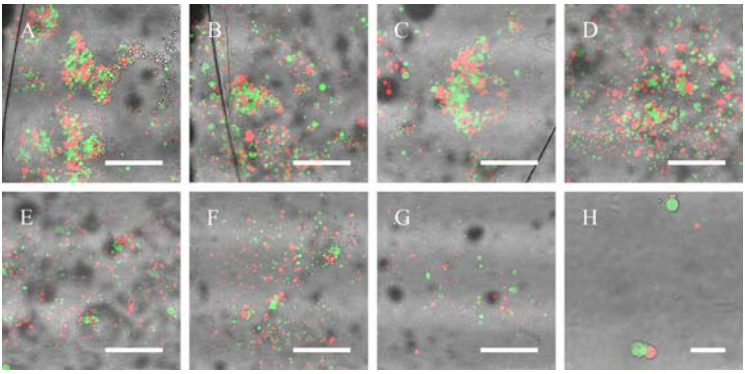


Fig. 40: GVs colonies formation from two population of POPC:oleate GVs labeled FITC or RITC-Dex at PLA concentration = 2.5ug/ml. GVs density is in A) = 10.000 GVs/ul; B) = 9.000 GVs/ul; C) = 8.000 GVs/ul; D) = 6.000 GVs/ul; E) = 4.000 GVs/ul; F) = 2.000 GVs/ul; G) = 1.000 GVs/ul; H) = 500 GVs/ul; Size bar (from A) to G)) = 250um. Size bar H) = 30um.

of GVs belonging to a colony. High GVs concentration brings about very large colonies, of about thousand GVs. By reducing the GVs concentration, smaller colonies are obtained, from composed by hundreds to tens vesicles, till very small colonies of just few units.
Furthermore, thanks to these experiments we also verified that the process of vesicle aggregation into colonies proceed in a reproducible and controlled way.

3.3.2 GVs fusion inside colony.

As we said earlier, the aim of these experiments with the colonies was to determine whether inside the colony GVs can strongly interact with each other in order to overcome the strong hydration forces that oppose the close approach between two vesicles and consequently fuse and/or exchange solutes (Figure 41). This would correspond to a stepwise increase of molecular complexity, from simple compartments to complex ones. Clearly the final goal would be the development of novel functionalities that do not exist in the single vesicles. To test GVs fusion, we have used two different populations of fluorescent giant fluorescent vesicles marked with two different fluorochromes, as in previous experiments. Colonies are formed from these two (or in some cases three) populations as described above. The fusion events were detected by co-localization of the markers within the same vesicle.

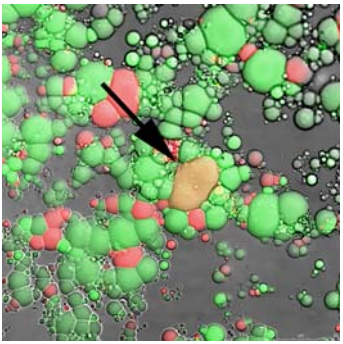


Fig. 41: Confocal image of POPC/Oleate GVs colony. The green ones are marked with FITC-Dextrane and the red ones with RITC-Dextrane. The arrow indicates a fused GVs. Its orange colour is the result of the co-localization of red and green ones. Size bar = 50um.

Regardless of the amount of PLA added or the number of GVs initially present in the microscopic slide well, it was always possible to detect 1-5% GVs that derive, without any doubts, from the fusion of two or more GVs. These GVs were not isolated, but belong to the colonies, so that we claim that their formation occurs within the colony, after the initial steps of colony growth. A typical example is shown in Figure 42.

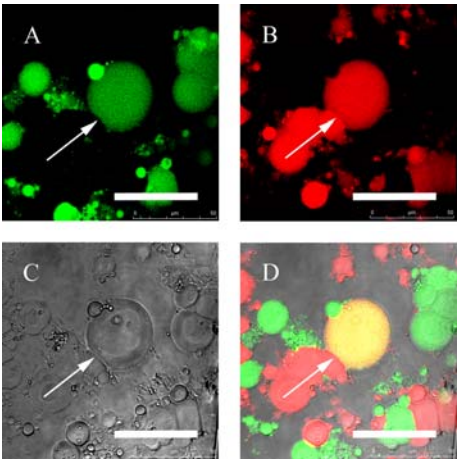
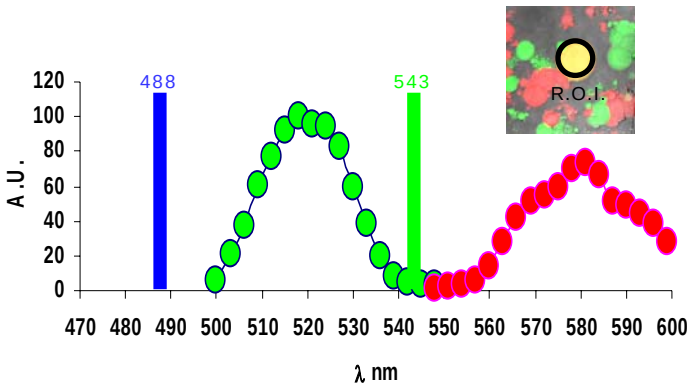


Fig. 42: Confocal image of fluorescent POPC:Oleate GVs colony. A) Green channel with FITC-Dexstrane; B) red channel with RITC-Dextrane; C) bright field image; D) overlapping of channels (A-C). The arrow indicates a fused GVs. Size bar = 50 µm.

Panel D of Figure 42 clearly show the overlap of green, red and bright light channels of the confocal micrograph, to give the orange color typical of co-entrapped FITC-dextran and RITC-dextran.

The co-localization of two fluorescent probes inside the fused GV is further confirmed by confocal spectral analysis. In fact, the lambda scan plot, obtained by scanning the region of interest (ROI) placed on the vesicle derived from fusion, reveal the emission peaks of both fluorocromes.



Graph 2: λ-scan graph obtained by placing the region of interest (R.O.I.) on the fused GVs inside the colony by confocal spectral analysis. Exciting the fused GVs with the laser 488, we observe the characteristic emission peak of fluorescein, while exciting it with the laser 543 that of rhodamine.

This first important result confirm that the colony formation brings about an increase of complexity, giving an advantage to the colony with respect to individual GVs. In fact, the two dyes are model of primitive functional solutes, that, being co-entrapped in the same compartment, after the fusion event, can further interact and give rise to richer reactive pathways.

Observing GVs colonies, it is clear that GVs are in close contact with each other. Their shapes is often not spherical as evident by the *xy* projection we see in confocal micrographs, but also they change shape in the *z* dimension (data not shown). We claim that the state characterized by closely packed membranes, very often found in GVs colonies, is the prelude to GVs fusion. It is reasonable to think that what we observe in GVs colonies is similar to what recently described by MacDonald (Pantazatos and MacDonald, 1999). In this article, the authors have studied the fusion mechanism of oppositely charged GVs, reporting the existence of *hemifusion* intermediates (Figure 42).

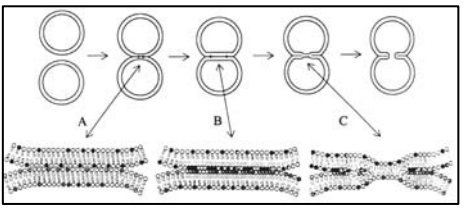


Fig. 42: Hypothesized interactions of oppositely charged lipid vesicles, from Pantazatos and MacDonald, 1999. For details see text.

According to their hypothesis, upon adhesion (stage II), in their case driven by electrostatic attraction, vesicles flatten against each other. Flattening requires an increase in the vesicle surface area, so that the membrane of each vesicle is stretched slightly, generating a tension in the vesicle bilayers. The tension so generated will oppose further adhesion and flattening, so that an equilibrium state can be attained (stage III). From this stage, in their case charge neutralization and accumulation (details A and B) in the contact zone reduces repulsion within each of the contacting outer monolayers. From this state the two outer monolayers can rend to give hemifusion (hemifusion; stage IV, detail C). From this state the system can remain stable or break under the combined tension of both vesicle membranes, leading to fusion (stage V). Therefore, the hemifusion/fusion processes are tuned by long range attractive force firstly, then to short-range lipid-lipid interaction, and final from forces that lead to fusion from the hemifusion intermediate. In the case of Pantazatos

and MacDonald (1999) the membrane composition in terms of molar fraction of charged lipids was the most important factor.
In our experiments we observe a similar pattern to that observed by the authors mentioned above.
In Figure 43, we report a series of images captured by real-time video-microscopy, at the time of formation of hemi-fusion intermediate starting from two single POPC:Oleate GVs, after PLA addition. After adhesion, GVs are flatten against each other and their

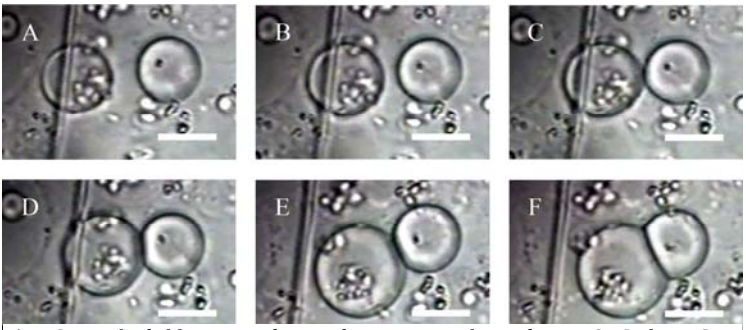


Fig. 43: Bright field images of Hemi-fusion intermediate of two POPC:oleate GVs. Each image was acquired every 500ms. Size bar = 10 µm.

shape is changed. At this point a hemi-fusion intermediate is formed, that can be stable as in this case, or lead to fusion. In the set of experiments carried out with video microscopy, it was not possible to record in real time any fusion event, even if we directly observed some fusion events via ocular observation. The low frequency of fusion occurrence limits the probability of capturing it by video-microscopy. We think that the formation of GVs colonies follows the principle of hemifusion, similarly to what observed by MacDonald and Pantazatos for oppositely charged GVs of opposite charge. In our case, anionic POPC:oleate GVs and cationic PLA attract each other and form stable structures via electrostatic bridges.
It is plausible to refer to the key intermediate as the localized arrangement of lipid bilayers and PLA on the flattened inter-GVs membrane region, where accumulation of oleate to the contact region cannot be excluded, due to the fluid nature of lipid bilayers. From this starting point, that is homologous to stage III of Figure 42, PLA could either: (*i*) favors the formation of non-lamellar inverse lipid phase, which are known to favor membrane fusion (Prestegard and O'Brien, 1987); (*ii*) pulls away the external membrane leaflets of the two GVs at the contact zone so that the hydrophobic tails of the

internal membrane leaflets can merge and bring about to the hemifusion step. From the latter, fusion could originate by the membrane tension.
One limitation of the GVs formed by emulsion inversion method is evident now. It is not easy to measure the concentration and the chemical composition of GVs produced. Quantitative chemical analysis is needed, and further work must be done to characterize GVs with respect to the lipids. Moreover, it is difficult to assess the real nature of the outer and inner membrane layers, i.e. the excess of one or of the other lipid (POPC and oleato) on each leaflet. Finally, it cannot be excluded that GVs produced by this method do not have the same lipid composition, e.g., some vesicle could have a higher or lower oleate content.
Therefore there could be oleate-rich GVs carrying a greater negative charge, or the contrary. As consequence we observe the simultaneous occurrence of (1) "no reaction"; (2) vesicle clusters – corresponding to (2a) just aggregated vesicles or (2b) to hemifusion intermediates; and (3) fusion. To date it is not easy to develop a model for vesicle aggregation and fusion, and further work is required.

3.3.3 Advantage to the colony with respect to individual GVs.
In addition to fusion and solute exchange, it was interesting to investigate additional advantages of GVs colonies with respect to individual GVs. When free GVs are on the bottom of the microscopic slide well, they can be washed away simply by aspirating the solution with a Gilson pipette. In contrary, after the addition of PLA and colony formation, the colony resists to water flow because it is strongly bound to surface. This behavior strongly resembles the formation of *biofilms* in bacteria. Bacteria take advantage of biofilms formation in several ways. In addition to mechanical resistance, they capture nutrients from the environment, are somehow protected from external agents (at least the inner elements of the biofilm) and can grow by capturing other bacteria, creating also complex colonies.
Inspired from this remarkable behavior, we tested whether GVs colonies can grow by incorporating new GVs or whether the colony is more stable than individual GVs against physical stress, like osmotic ones.

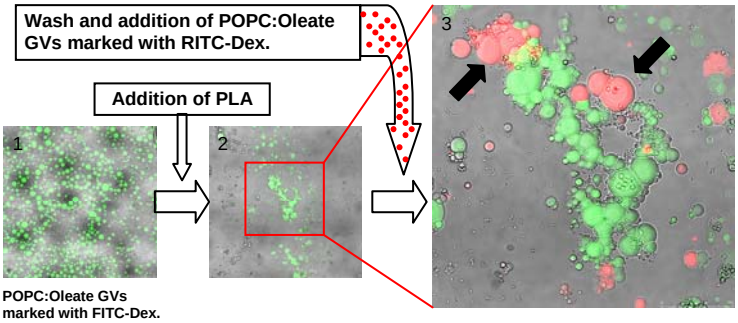


Fig. 44: GVs colony resist to water flow and addition of new Giant Vesicles brings about colony growth. In the first confocal image, there is a population of POPC:Oleate Giant Vesicles marked with FITC-Dex. After the addition of PLA giant vesicles join to form a colony (2). At this point, three washes with fresh buffer were made and an other population of POPC:Oleate Giant Vesicles marked with RITC-Dex was added. As you can see in the third image, not only is the colony not washed away, but some of the new vesicles are attached to the edge of the colony as indicated by arrows.

In the first experiment, GVs colonies were created from PLA and POPC:oleate GVs, marked with green-fluorescent FITC-Dextran. GVs colonies, as stated before, firmly adhere to the well and therefore can be washed, so that free GVs and excess PLA are removed. After the addition of a fresh GVs population (POPC:oleate GVs marked instead with RITC-Dextran), it was successfully observed the growth of the colony (Figure 44). Remarkably, some of the new red-fluorescent vesicles are attached to the edge of the colony as indicated by arrows.

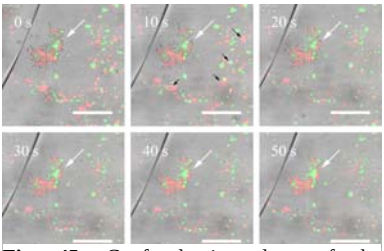


Fig. 45: Confocal time laps of the experiment obtained at 80mM of NaCl. After the addition of NaCl, GVs colony has resistance to osmotic stress, as indicated by white arrow, while many of the single GVs burst, as indicated by black arrows. Confocal images were captured every 10s.

It is noteworthy that the excess of PLA was washed away, so the new GVs are attached to the colony only thanks to the residual fraction of PLA still available on the external colony surface. In the second experiment, we tested whether and at what extent single GVs and GVs colonies can resist to physical stress. We have tested this behavior by putting in the same slide well both colonies

and single vesicles, and adding various concentrations of NaCl starting (from 30 to 500 mM). The time-dependent behavior was recorded by time-lapse confocal microscopy (Figure 45).
We have surprisingly observed that the colony can resist up to a concentration of 125 mM NaCl, while the single vesicles already burst at 60 mM.
This shows that GVs colonies are more stable than free GVs with respect to hypertonic stress. In contrary, we have not observed significant differences in the behavior of free GVs and GVs colonies against hypotonic stress (i.e., both are equally resistant). In Table 25 a summary of the resistance (v) and susceptibility (x) of GVs colonies and free GVs.

Tab. 25: Results about time laps by confocal microscopy about resistance to osmotic stress using NaCl. GVs colonies have resistance up to a concentration of 125 mM of NaCl, while the single GVs already burst at 60 mM.

[NaCl] mM	Colony	GVs
30	✔	✔
60	✔	✘
80	✔	✘
125	✔	✘
250	✘	✘
500	✘	✘

Chapter 4. Conclusions and prospectives

This PhD is part of a broader research program called “The Minimal Cell” with relevance in the Origin of Life studies and in Synthetic Biology. The Minimal Cell program aims to build a semi-synthetic cell in a laboratory, by means of constructing cell models having the minimum and sufficient number of components to define "alive", then capable of self-maintenance (homeostatic metabolism), self-reproduction and capacity to evolve. The experimental approach involves the design and realization of complex cell biology reactions within synthetic compartments as lipid vesicles (liposomes). These attempts, clearly at the beginning of an ambitious research program, will help in future development, hopefully bringing to the first living-like compartment build in the laboratory. The international interest in these issues demonstrates that the "Minimal Cell" project is considered a target experimentally accessible, albeit not in the short term.

GVs are useful models for the study of cells. They are closed compartments with a lipid membrane boundary, which separates an interior content that can be very different from the external environment. Thanks to their semi-permeable membrane, GVs can exchange solutes with the environment. Most importantly, GVs have size between 1 and 100 um, which allow their direct observation by light microscopy. Thanks to all these features, that make them comparable to the actual living cells, GVs are very valuable models for developing cell models for ambitious projects as the “Minimal Cell” one.

The most promising method for the GVs production, from the viewpoint of developing a robust technology of cell model construction, is the emulsion inversion (or droplet transfer).

The first important result of this PhD work is the efficient and reproducible production (10,000 GVs/ul) of GVs with emulsion inversion method. In other words, a general protocol was developed in our laboratory. This will be the subject of a publication in the near future. GVs have been produced from different lipid systems, by using mixtures of two kinds of lipids, a zwitterionic phospholipids (POPC) together with a charged lipid. This was the key step for obtaining a very effective GVs production. Anionic GVs can be prepared from POPC (used as emulsifying agent) and POPG or POPS (negatively charged phospholipids), as well as with sodium

oleate (negatively charged fatty acid). Cationic GVs can be prepared from POPC and DDAB or PUCE (positively charged synthetic surfactants). A series of water-soluble fluorescent markers of different molecular size and different chemical structure, such as calcein (a small soluble molecule), FITC and RITC-dextran (polysaccarides), phycoerythrin and allophycocyanin (proteins), were effectively entrapped inside GVs. Current experiments are devoted to the entrapment of nucleic acids (data not shown). The protocol developed in our laboratory derives from a series of optimization experiments carried out by varying all variables affecting the quantity and the quality of produced GVs, joined by quantitative image analysis.

In addition to the production method, the second important result is related to the study of the mechanism of GVs formation. It is fair to say that this has not been completely understood, and further work will be devoted to clarify the details as much as possible. Our working hypothesis stems from the consideration that high GVs productions are always obtained when a charged lipid was placed at the oil/water macroscopic interface. In particular, by varying the amount of charged lipid at the interface (POPC:oleate at different ratios), it is shown that without oleate – the charged component, the production of GVs is almost inhibited. In fact, real-time video-observation of the transition of w/o droplet through the interface revealed that neutral interfaces rapidly become depleted of surfactant, which form local aggregates and/or w/o droplets. In contrary, interfaces created by charged lipids are smooth and sustain the passage of w/o droplets to form GVs. We have explained this behavior by suggesting that oleate is able to quickly establishing a homogeneous interface, due to its higher dynamical pattern, which is known from aqueous systems, and thanks to higher affinity of the charged head group for the water side of the interface.

These results represent a starting point in the next research of "Minimal Cell" project; thanks to this new protocol we will able to entrap many components within a single GVs and you be therefore able to implement basic metabolic pathways or protein expression in GVs.

In particular, we could entrap the whole protein expression kit (commercially available), and verify the production of a protein, e.g. a fluorescent protein. At this point, taking advantage of the large size, it becomes possible to observe, in real time, the protein

production in each GV; and possibly correlate the internal reactivity to their content (a second fluorescent probe can be co-entrapped). This approach is not yet present in the literature.
The third result of this work is the direct observation of vesicle fusion. We believe that this process could have had a key role in the origin of life: in fact, it is difficult to imagine that the first cells have originated with all the components necessary for life inside a single compartment, and therefore a possible new vista involves the consideration of community of individuals, capable of communicating in chemical way and exchange solutes. Our strategy was to aggregate oppositely charged GVs (e.g., negatively charged POPC:POPG with positively charged POPC:DDAB) and to verify, via confocal laser scanning microscopy, if spontaneous fusion between two or more GVs occurs. This is possible by co-localization analysis of two fluorophores, initially present in two different GVs populations.
Preliminary results indicated that GVs fusion was a non-trivial process, in fact, no results on the spontaneous GVs fusion are present in the literature. According to our analysis, this is a difficult step because GVs' collision frequency is quite low, and also their low-curvature membrane is highly stable. We made the hypothesis that fusion would have been much more probable if GVs were in close contact. Interestingly, this approach correspond to a formation of a GVs colony, where individuals are tightly bound and membrane fusion can occur. In order to create a GVs colony, we simulated the presence of primitive bridging agents by using a simple cationic polypeptide, i.e., poly(arginine) (PLA) and anionic GVs, formed by POPC and oleate.
The aggregation between anionic vesicles triggered by PLA has been studied in detail by varying the PLA concentration (2.5-100 ug/ml) and the GVs number. The evaluation of these factors allows the creation of GVs colonies of different size in a reproducible and controlled way.
Furthermore, regardless of the colony size and of the PLA concentration used, a spontaneous fusion of some GVs (1-5%) within colonies has been always observed (as demonstrated by co-localization of FITC and RITC-Dex into a single fused GV). GVs fusion represents a very valuable process that allow two or more GVs exchange and mix their content so that different reactants,

initially separated in different compartments, enter in contact and possibly react to give rise to interesting behaviors.
We have therefore correctly identify a plausible path from isolated vesicles to GVs aggregate (GVs colony) to GVs fusion.
As fourth result, after verifying that GVs fusion really occurs within a colony, we asked whether the colony could have a selective advantage with respect to individual (free) GVs. GVs colonies resulted to bevery stable, they strongly adhere to the support and are able to withstand an aqueous flow (while the individual GVs are washed away). GVs colonies have a higher resistance (up to 125 mM) in respect of single GVs (60 mM). Moreover, GVs colonies can grow by capturing new GVs so that the colony is not a dead-end product of aggregation by can be a starting point for successive transformations.

In conclusion, this work has opened a new perspective with respect to phenomena possibly important for the origin of life. It is the first time in experimental work that the vision of a single compartment is abandoned in favor to that one of a community. A colony of individuals working together can beable resist better to environmental changes, develop new reactive patterns by fusion and/or solute exchange, and ultimately can have selective advantages in respect to the individuals, and thus evolve and achieve a greater level of complexity. We also think that this model of primordial cell community is a more realistic model for simulating the reality of early cellular pre-life.
This new approach excites the imagination and surely will be developed in future experiments. First it should be checked if this aggregation process occurs by using other peptides, and GVs with a membrane made from other lipids. So, for example, use positively charged GVs and negatively charged peptides. It could be designed selection experiments using two or more kinds of GVs against a peptide, or on the contrary, several kinds of peptides with a single GVs population. And yet, try to form GVs colonies in three dimensions, or to grow the colonies and then split them.
In order to make a realistic model, GVs fusion could be checked not only by fluorophore co-localization, but by developing enzyme/substrate reactions, from two different GVs population, one containing the enzyme and the other the substrate. The next step

would be the creation, possibly stepwise, of simple metabolic routes based on several enzymes.
It would also be interesting to see if this aggregation process occurs with conventional liposomes (submicrometric) and compare the properties of micro-colonies with those formed by GVs.
And finally, GVs colonies can be extensively tested again a set of different environmental stresses, both physical (heat, temperature cycles, aqueous flow) and chemical (pH change, resistance to surfactants and expand the study on osmotic stress with other kinds of molecules), as well as studying their behavior over time thanks to the fact that they are fixed to the support and therefore be traced in time one by one.
In other words, GVs colonies must be characterized as an example of supramolecular structure of higher hierarchy, namely an assembly of GVs, which in turn are assemblies of lipids. This stimulates novel experimental and theoretical investigations in a really new field related to cell models in origins of life and synthetic biology.

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Publications

Carrara P.; Stano P., Mavelli F., Caputo M. and Luisi P.L., Statistical analysis of EGFP expression in a population of water-in-oil droplets. Manuscript in preparation, to be published in a peer-review biophysical journal by the end of 2010.

Carrara P., Stano P. and Luisi, P.L., Giant vesicle formation by droplet transfer method. A review. Manuscript in preparation, to be published in a peer-review biophysical journal in the first months of 2011.

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Materials and methods

List of reagents used:

Phospholipids: POPC (1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylcholine); POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol) and POPS (1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylserine) (Avanti).

Fatty acids: sodium oleate (Fluka).

Cationic surfactant: DDAB (didodecyl-dimethyl-ammonium bromide (Fluka); PUCE (palmitoyl-undecylcarnitine –ester, chloride salt) (courtesy of Sigma-Tau, S.p.A.).

Buffers: Tris HCl (Sigma).

Fluorescent markers: calcein (calcein disodium salt) (Fluka); RITC-dextran (Rhodamine B isothio-cyanate-Dextran), FITC-dextran (Fluorescein isothio-cyanate-Dextran), allophycocyanin (APC), phycoerythrin (PE) (Sigma).

Others reagents: D-(+)-glucose (sigma); D-(+)-sucrose (Fluka); Mineral Oil M5904 (Sigma).

Equipments:
Microscopic slides: Bright-Line Hemacytometer (Hausser Scientific) was used for GVs counting. IBIDI sterile micro-wells slides µ-Slide 8 well, µ-Slide 18 well-flat and µ-Slide VI$^{0.4}$ uncoated were used to study GVs colonies.

Microscopy.
GVs were observed by using: (1) an Olympus BX 51 light microscope equipped with Leica DFC 420 camera; (2) a Leica TCS SP5 confocal inverted microscope, with Application Suite acquisition system; (3) an inverted Zeiss Axiovert 25 microscope with Hamamatsu C5810 real color digital videocamera and videorecorder.

Software (image analysis).

Image J free software.

Preparation of lipid solutions in mineral oil.
The exact amount of lipid powder was weighed in order to obtain lipid solutions at a concentration of 1mM.
POPC: M.W. = 760g/mol;
POPG: M.W. = 697g/mol;
POPS: M.W. = 760g/mol;
oleate: M.W. = 305g/mol;
DDAB: M.W. = 463g/mol;
PUCE: M.W. = 590g/mol.
The dissolution of lipid in mineral oil was achieved by magnetic stirring for 2 to 3 days at 4°C. The resulting lipid solutions should not be cloudy. Solutions can be stored in refrigerator at 4°C for weeks without oxidation.

Preparation of aqueous buffers.
Outer buffer: Tris HCl 10mM / glucose 100mM / pH 7.5.
Inner buffer: Tris HCl 10mM / sucrose 100mM / fluorescent marker (calcein 10um / FITC/RITC-Dex both at 0,5mg/ml) pH 7.5.

GVs emulsion inversion preparation method.
In a eppendorf tube (1.5 mL) were put in the following order: 500ul external (outer) buffer, then, gently, 500ul of 1 mM charged lipid solution in mineral oil (POPG, POPS, oleate, PUCE, or DDAB) to create the oil-water interface and incubate at RT for 1h. Prepare the water in oil emulsion by adding 10uL of inner buffer to 500ul of a 1 mM POPC in mineral oil(emulsion 2% v/v). The emulsion is formed by pipetting with a Gilson micropipette, until obtain a cloudy emulsion is obtained. Visible droplets should not be visible to the naked eye. The w/o emulsion (500 ul) was then gently poured above the interface phase and the tube was immediately centrifuged at 2000rpm for 10 minutes at RT. After centrifugation, mineral oil is removed, and the aqueous phase is centrifuged again at 5000rmp for 10 minutes at RT. A small pellet composed of GVs is observed at the bottom of the eppendorf tube.

GVs washing.
During the formation process, many GVs are broken during the passage through the interface releasing their contents into the

external buffer. Also, residual mineral oil is often present. In order to eliminate the oil and released markers Gvscan be washed with fresh external buffer. GVs are washed by gentle aspirating the GVs pellet with a Gilson micropipette. Typically, 200ul including the pellet is taken. Mix the aspired GVs (200ul) and 300ul of fresh outer buffer in a new eppendorf tube. Then centrifuge at 5000rpm for 10 minutes at RT. A pellet at the bottom of the eppendorf should be obtained again. Repeat the washing procedure again. If needed, GVs can be washed further, by repeating the washing procedure several times. The GVs concentration in the final solution can be changed by using smaller or larger volumes outer buffer in the washing steps.

GVs counting.

To verify the number of GVs obtained in the preparation, take a representative 10uL GVs sample (by pipetting several times), and place itin a hemacytometer slide. Place the slide on the microscope and wait at least 10 minutes to allow GVs sedimentation. Then capture 10 fluorescent picture, moving at random on the slide, but being careful to maintain always GVs in focus at the bottom of the slide. GVs are visualized by fluorescence, so that the GVs must be prepared with a fluorescent probe inside. In fact, being almost transparent, it is almost impossible to count them in bright field. Depending on the objective used, the picture has a definite *xy* size, whereas the *z* dimension is set to 100um (standard dimension of slide well). Consequently, therefore the volume corresponding to each micrograph is known. Images are analyzed by using ImageJ software in order to get the vesicle number and diameters (size distribution), from which the average diameter and GVs volume are estimated. Calculations have been performed by means of MS Excel spreadsheets.

GVs colonies formation.

For the formation of GVs colonies the following microscope sterile IBIDI slides (Germany) were used: (1) µ-Slide 8 well (500ul); (2) µ-Slide 18 well - flat (30ul); and (3) µ-Slide VI$^{0.4}$ (100ul) uncoated.. Depending on the slides, 180, 27 or 90ul solution GVs were respectively used. The GVs were allowed to sediment on the microwell for 10 minutes, by placing the slide on the microscope stage. PLA was then added (20, 3 or 10uL, respectively) by a

micropipette, from stock solutions (25ug/ul to 1 mg/ml). In this way the PLA final concentration in the well is between 2.5 and 100ug/ml.