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# Exploring Giant Unilamellar Vesicle Production for Artificial Cells – Current Challenges and Future Directions

Lori van de Cauter, Lennard van Buren, Gijse H. Koenderink,\* and Kristina A. Ganzinger\*

Creating an artificial cell from the bottom up is a long-standing challenge and, while significant progress has been made, the full realization of this goal remains elusive. Arguably, one of the biggest hurdles that researchers are facing now is the assembly of different modules of cell function inside a single container. Giant unilamellar vesicles (GUVs) have emerged as a suitable container with many methods available for their production. Well-studied swelling-based methods offer a wide range of lipid compositions but at the expense of limited encapsulation efficiency. Emulsion-based methods, on the other hand, excel at encapsulation but are only effective with a limited set of membrane compositions and may entrap residual additives in the lipid bilayer. Since the ultimate artificial cell will need to comply with both specific membrane and encapsulation requirements, there is still no one-method-fits-all solution for GUV formation available today. This review discusses the state of the art in different GUV production methods and their compatibility with GUV requirements and operational requirements such as reproducibility and ease of use. It concludes by identifying the most pressing issues and proposes potential avenues for future research to bring us one step closer to turning artificial cells into a reality.

nonliving molecular assemblies can give rise to living cells and what life truly is,<sup>[1]</sup> has sparked great interest for many decades. The concept of rebuilding life from its molecular components<sup>[2]</sup> has led to the development of synthetic cell research, a new interdisciplinary scientific field, synchronized with the establishment of various national and international research initiatives to further the goals of recreating life<sup>[3]</sup> (such as BaSyC in the Netherlands,<sup>[4]</sup> MaxSynBio in Germany,<sup>[5]</sup> fabriCELL in the UK,<sup>[6]</sup> Build-a-Cell community in the USA,<sup>[7]</sup> and the European Synthetic Cell Initiative in Europe<sup>[8]</sup>). Despite significant research efforts and a widespread public interest, the recreation of life in the lab in the form of an artificial cell remains an immensely challenging task that is still in its early stages today.


Generally, there are two possible strategies to constructing an artificial cell (Figure 1). The classic biological approach (also called a top-down or in vivo

reductionist approach), which involves modifying components in their biological context, has provided valuable insights in vital genes,<sup>[9,10]</sup> but has limitations in unraveling mechanistic working principles. The biological context is so overwhelmingly complex (human cells have 20 000 genes,<sup>[11]</sup> and typical number density is 0.2–4 million protein molecules per cubic micron<sup>[12]</sup>) that it is generally challenging to unequivocally attribute a particular function to a single component. Even more difficult than understanding how single components function within the cell, is to understand how cooperation between molecules can give rise to complex emergent processes such as cell division and migration.<sup>[13]</sup> A top-down approach thus offers limited understanding of how life is assembled from its nonliving parts.<sup>[14]</sup> Instead, to obtain a mechanistic understanding of cellular parts and processes, it is often necessary to isolate the components of interest and study them in a well-defined chemical environment.<sup>[15]</sup> This bottom-up or constructive approach has gained significant traction over the past three decades and, in concert with technological advances, scientists have been aiming to reconstitute cellular processes of increasing complexity.<sup>[13,16]</sup> Typically, the aims of this bottom-up strategy are twofold: on the one hand the focus is on acquiring fundamental knowledge on the building blocks of life and how they interact, while on the other hand, efforts are directed toward engineering a fully

## 1. Giant Unilamellar Vesicles in the Artificial Cell Landscape

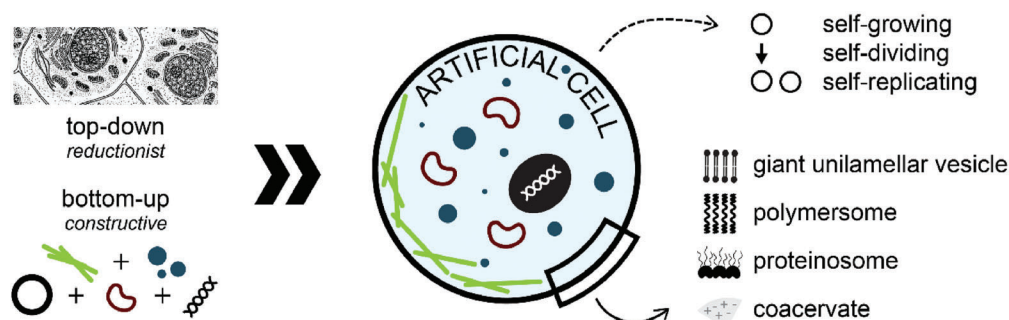
Cells are the smallest units considered to be alive and in turn the building blocks for other, more complex, living organisms. Despite being the smallest unit of life, they exhibit a bewildering complexity at the molecular level. The questions of how those

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**Figure 1.** Giant unilamellar vesicles in the artificial cell landscape. Schematic representation of the different strategies for constructing an artificial cell. A top-down approach involves modifying components in their biological context, while a bottom-up approach relies on the stepwise integration of different building blocks in a well-defined chemical environment. The aim is to construct an artificial cell, which we define as a self-growing, self-dividing, and self-replicating entity. Compartmentalization is a crucial step in the construction of such an artificial cell, and possible strategies involve the use of giant unilamellar vesicles with a lipid bilayer, polymersomes, proteinosomes, or coacervates as the container.

artificial cell. The general strategy here involves enhancing complexity through the stepwise integration of different building blocks or modules in both time and space, eventually recreating complex dynamic processes like cell growth, replication, and division. At the same time, reconstituted systems of intermediate complexity hold potential in for example medical applications, e.g. as artificial blood cells or for drug delivery.<sup>[17,18]</sup> In this review, we will think of an artificial cell as a self-growing, self-dividing, and self-replicating entity, constructed from the bottom up by integrating the various components needed inside a compartment (Figure 1).

An essential step for building artificial cells from the bottom up is hence the construction of a “container” for its components. Compartmentalization is not only a fundamental feature of life but is often also considered to be essential for enabling life and its out-of-equilibrium chemistry in the first place.<sup>[19]</sup> The molecular content of an artificial cell thus needs to be contained within an aqueous compartment, in which all reconstituted cellular processes can take place. To allow for growth and division, this compartment should also have a deformable envelope. While proteins and metabolites stay confined, the container should allow for selective import of nutrients and export of waste products to grant survival. Structurally, a large array of design strategies for creating such compartments exists, all offering different benefits and drawbacks.<sup>[20]</sup> Possible strategies include membraneless compartments (coacervates<sup>[21]</sup>), compartments confined by membranes composed of polymers (polymersomes<sup>[22,23]</sup>), proteins (proteinosomes<sup>[24]</sup>), or lipids (liposomes<sup>[25–27]</sup>), or hybrid approaches.<sup>[28,29]</sup> Given that all life as we know it is compartmentalized by lipid membranes across all kingdoms of life,<sup>[30,31]</sup> liposomes are the closest mimic to biological cells and therefore ultimately the best choice as the compartment for an artificial cell made to closely mimic its biological inspiration. The use of liposomes also ensures compatibility with other biological building blocks, such as membrane-bound enzymes and transporters. Moreover, the size of giant liposomes, or giant unilamellar vesicles (GUVs), is similar to the size of eukaryotic cells (5–100 μm).<sup>[25]</sup> These cell-like properties and their biological compatibility make GUVs the perfect chassis for building an artificial cell and, unsurprisingly, GUVs have already been used for a wide

range of research applications in biophysics, biomedicine, and synthetic biology.<sup>[25]</sup>

## 2. GUVs as the Container for Artificial Cells—What Are the Requirements?

To achieve the properties and functions desired for an artificial cell, GUVs must satisfy a broad range of requirements (Table 1). A first important criterion to consider is size and the ability to tune the final size as desired. The GUV size sets the degree of confinement and surface-to-volume ratio, which in turn influence growth (via lipid production), division (via establishment of cell polarity by reaction-diffusion and/or cytoskeletal systems), and replication (via energy metabolism).<sup>[32]</sup> Further requirements can be divided into two categories: the requirements for the container itself, i.e., the membrane surrounding the aqueous solution, and the requirements for the lumen, i.e., the encapsulation of different components.

Lipids are a highly diverse group of biomolecules with varying structures and properties.<sup>[33,34]</sup> While they all share the common property of having a hydrophilic head and two hydrophobic tails, which ensures their self-assembly into bilayers, they vary in head group size and charge (dependent on pH), length of hydrocarbon tails, and saturation of the tails. As such, lipid properties regulate interactions within the bilayer, thereby determining membrane elasticity and fluidity, as well as interactions with the external environment, like electrostatic interactions with proteins and ions. An important property arising from the lipid structure is its molecular shape, or intrinsic curvature, which determines the spontaneous curvature once assembled into mono- or bilayers.<sup>[35,36]</sup> Lipids with cylindrical molecular shapes lead to the formation of flat bilayers, while conical-shaped lipids lead to curved membranes. Certain lipids in cell membranes play important roles in the function of membrane proteins, anchoring of cytoskeletal proteins, or in signaling. Examples are phosphatidylserine, which interacts with many proteins via electrostatic interactions, and phosphoinositides, which play a prominent role in signaling processes.<sup>[37,38]</sup> In addition to lipids, biological membranes also contain other important

**Table 1.** Overview of requirements for GUVs as the container of an artificial cell.

Category	Requirement	Considerations	
General	Size	5–100 $\mu\text{m}$ range	
		Size control	
Membrane	Composition	Synthetic lipids	
		Natural lipids	
		Cholesterol	
	Functionalization	Functionalized lipids	
	Compositional asymmetry		
	Controlled permeability	To allow deformability for growth and division	
	Protein reconstitution	Transmembrane proteins	
	Unilamellarity	For protein reconstitution	
		Mechanical properties	
		For permeability control	
Cleanliness		Absence of residual oil and other inclusions	
	Mechanical stability	For manipulation, observation, and longevity	
	Encapsulation	Physiological buffers	Physiological ionic strength (50–150 $\times 10^{-3}$ M) Absence of auxiliary molecules
		Efficiency	
Complex reconstitution	Multiple components in right stoichiometry		
Cross-compatibility	Different biological systems within a single GUV		

molecules such as cholesterol in animal cells or hopanoids in bacteria.<sup>[39]</sup> Cholesterol, a hydrophobic organic molecule with a small hydrophilic head group, is a key component of animal cell membranes as it integrates into the bilayer where it modulates lipid packing, thereby controlling fluidity, permeability, and elasticity (reviewed in refs. [40,41]). Being able to use a large range of lipids, including synthetic and natural lipids, and the option to include cholesterol is therefore crucial for producing a GUV-based artificial cell. Synthetic lipids can further be functionalized with a variety of synthetic polymers or interacting groups, e.g., poly(ethylene glycol) (PEG) linkers, fluorescent labels, nickel chelating groups, or azobenzene moieties. This functionalization can provide additional properties to the membrane, such as reducing nonspecific interactions,<sup>[42]</sup> generating photoswitchable lipids,<sup>[43]</sup> selective anchoring of molecules or proteins to the inner or outer leaflet, and more.

In biological membranes, the bilayer is compositionally asymmetric, with the inner leaflet being different in lipid and protein composition from the outer leaflet. This transmembrane asymmetry is vital for cell signaling, functioning, differentiation, and growth<sup>[44]</sup> and occurs because lipids cannot easily transfer from one leaflet to the other,<sup>[45]</sup> a process known as flip-flop. In turn, compositional asymmetries give rise to spontaneous curvature effects, which in protein-free lipid membranes are known to lead to membrane deformations such as lipid nanotubes.<sup>[46]</sup> Besides compositional asymmetry, this spontaneous curvature can also be generated by an asymmetry of the presence of membrane-interacting solutes such as sugars,<sup>[47]</sup> ions,<sup>[48]</sup> and proteins.<sup>[49]</sup>

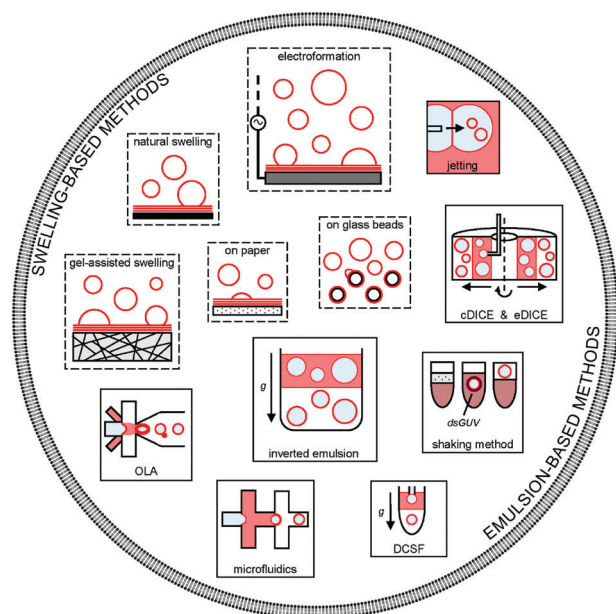
Mimicking the asymmetrical bilayer composition of biological membranes in GUVs is thus a way of enhancing complexity and transcending to more biologically relevant compositions, necessary for, for example, reconstituting cell–cell interactions.

Lipid bilayers are permeable to hydrophobic molecules and small uncharged polar molecules like water, but not to protons, ions, and large uncharged polar molecules like sugars and proteins.<sup>[50,51]</sup> This permeability can be modulated by altering the lipid composition, providing a means to fine-tune its properties.<sup>[52]</sup> As water can move across the membrane but solutes cannot, GUVs are subject to osmosis. In vitro, controlled osmosis allows for deformability and control of excess membrane area and membrane tension. In vivo, permeation of ions and large molecules is facilitated by membrane proteins such as transporters and channels.<sup>[53,54]</sup> For the construction of a GUV-based artificial cell, it would thus be desirable to have the ability to incorporate membrane proteins into GUVs.<sup>[55]</sup> To achieve this, a unilamellar membrane, and the absence of residual solvents or additives, is of particular importance.<sup>[55]</sup>

The unilamellar lipid bilayer furthermore affects the mechanical properties of GUVs, which determine the possible shape transformations and mechanical stability. The mechanical properties of GUVs are influenced by a range of physical parameters, including the membrane's bending rigidity, stretching modulus, and tension.<sup>[56]</sup> Membrane “cleanliness”, alluding to the membrane being composed of a controlled number of bilayers, with no entrapments, residual oil, or other inclusions altering the above-mentioned criteria, is an especially important criterion to consider with regard to mechanical properties. The mechanical properties of GUVs are crucial for the successful reconstitution of processes that affect its size and shape (e.g., growth and division) as well as for the ease of experimental manipulation, observation, and longevity. However, GUV membranes are naturally unstable and lack robust mechanical properties as compared to their natural counterparts, cell membranes. As reviewed in Wubshet et al.,<sup>[57]</sup> GUV stability can be enhanced by membrane modulation, e.g., by tuning the lipid composition, changing bilayer asymmetry, or altering the cholesterol concentration, or by luminal modulation, e.g., by encapsulating cytoskeletal components or other, structurally relevant molecules.

So far, we discussed the membrane requirements for GUVs, yet the membrane only comprises half of the challenge. Requirements concerning the lumen of the GUV, or encapsulation of molecules, are equally important. GUVs must be able to encapsulate physiological buffers ((50–150)  $\times 10^{-3}$  M) in the absence of any type of chemical that can interfere with biological processes. Furthermore, it is important to have the ability to achieve complex, multicomponent reconstitutions in which different types of proteins or protein machineries are efficiently encapsulated in the right stoichiometries. Moreover, emphasis should be placed on cross-compatibility, i.e., the ability to encapsulate diverse biological systems in a single GUV, which are often reconstituted under different buffer conditions. An ideal system should thus be able to accommodate multiple biological systems with ease, with a minimum of auxiliary molecules that may hinder their normal functioning.

In essence, producing GUVs for artificial cells thus requires meeting two main goals: having a complex and biologically analogous membrane while achieving a multicomponent lu-



**Figure 2.** Overview of GUV production methods. Generally, there are two different approaches to GUV formation. Swelling-based methods rely on natural hydration of a lipid film, while emulsion-based methods are based on the passage of a water-in-oil emulsion through an oil–water interface for bilayer formation. Swelling-based methods, indicated by the dashed lines, include natural/spontaneous swelling, gel-assisted swelling, swelling on various porous substrates such as glass beads or textile/paper, and electroformation. Emulsion-based methods, indicated by the solid lines, include inverted emulsion, microfluidic-based methods (e.g., octanol-assisted liposome assembly (OLA) and other PDMS-based microfluidic methods), jetting, continuous/emulsion droplet interface crossing encapsulation (cDICE/eDICE), the shaking method that forms GUVs via intermediate droplet-stabilized GUVs (dsGUVs), and droplet-shooting centrifugal formation (DCSF).

men. Careful consideration of these requirements is necessary to produce GUVs that can accurately mimic the physiological environment of a cell, and consequently serve as a suitable container for an artificial cell.

### 3. Overview of Available Methods for GUV Production

Over the past 50 years,<sup>[58]</sup> numerous methods for producing GUVs have been developed.<sup>[25,59]</sup> Currently, more than twenty different techniques exist, giving offspring to hundreds of different protocols, ranging from relatively straightforward bulk techniques to sophisticated microfluidic assembly lines.<sup>[16,57,60,61]</sup> GUV production methods are typically classified into two main categories: swelling-based approaches, which rely on rehydrating dried lipid bilayers, and emulsion-based approaches, where lipids are initially adsorbed to water–oil interfaces and a bilayer is only formed after subsequent transfer through an oil–water interface (Figure 2).

Originally proposed swelling methods involved hydration of a lipid film in aqueous environment, commonly referred to as natural or spontaneous swelling or gentle hydration.<sup>[58]</sup> Later, Angelova et al.<sup>[62]</sup> showed this natural swelling process could

be accelerated by the application of an alternating electric field, leading to the development of electroformation. Historically, swelling-based approaches have been used widely in the biophysical community to study membrane biophysical properties outside of any cellular context, e.g., bilayer elasticity,<sup>[63,64]</sup> lipid diffusion,<sup>[65,66]</sup> membrane lateral organization,<sup>[67]</sup> and membrane permeability.<sup>[68]</sup> While electroformation has been the gold standard for GUV formation since its invention, a major disadvantage has been the limited compatibility of this method with charged lipids and physiological buffers. However, widespread research efforts have meanwhile led to the successful formation of GUVs in a wide range of buffer compositions while also incorporating a broad range of lipids, discussed in more detail in Rideau et al.<sup>[61]</sup> and Boban et al.<sup>[69,70]</sup> Alternatively, porous hydrogels naturally promote GUV swelling in solutions of higher ionic strength.<sup>[71–74]</sup> However, gel-swollen GUV membranes can be contaminated with gel polymers, thereby leading to altered membrane properties.<sup>[75]</sup> To produce clean membranes, efforts have been concentrated on changing the physicochemical properties of the hydrogels,<sup>[71]</sup> cross-linking the polymers,<sup>[76]</sup> or using other (porous) substrates such as glass beads,<sup>[77,78]</sup> and more recently, textile,<sup>[79,80]</sup> and paper (PAPYRUS).<sup>[81,82]</sup> Although these more recent techniques offer welcome simplicity and make GUV formation more accessible to a wider audience, they are still in their infancy. Altogether, a wide set of swelling-based GUV formation methods has been developed to produce GUVs with membranes of varying compositions, in buffers of different ionic strength. Notwithstanding these valuable results, swelling-based techniques generally only have limited compatibility with more complex biological reconstitution experiments as efficient encapsulation of large and charged water-soluble molecules remains hard to achieve using these methods.<sup>[25]</sup> This includes proteins, a synthetic genome, and other biomolecules that form the basis for the cell-mimicking content of artificial cells.

When complex encapsulation is required, researchers often resort to GUVs formed from emulsion droplets by emulsion-based techniques.<sup>[83]</sup> Here, water droplets are formed in an oil phase containing lipids, which adsorb to the droplet water–oil interface to create a lipid monolayer (i.e., water-in-oil emulsion). Next, after transfer of these droplets through another oil–water interface, likewise covered by a lipid monolayer, the droplets acquire a second lipid monolayer and transform into GUVs. As opposed to swelling-based methods, emulsion-based methods thus make use of solvent-displacement. This can lead to the undesirable entrapment of residual additives in the final lipid bilayer, thereby possibly altering the mechanical properties of the GUV,<sup>[25]</sup> which is often regarded as the most significant drawback of emulsion-based methods. However, owing to this two-step solvent displacement process, emulsion-based methods do offer the ability to control the inner aqueous phase independently from the outer aqueous solution, and furthermore allow assembly of the lipid monolayers one by one, enabling the assembly of asymmetric bilayers.<sup>[84–87]</sup> Despite the introduction of inverted emulsion just fifteen years ago, and the even more recent emergence of various related methods, emulsion-based methods have already allowed numerous advancements in complex reconstitution experiments in this comparatively short period.<sup>[83,88–90]</sup> Furthermore, the working principle of inverted emulsion is suitable for automation and has given rise to a plethora of

**Table 2.** Overview of compatibility of different GUV production methods with membrane requirements based on state-of-the-art results.

	Method	Size <sup>a)</sup>	Compositional complexity						Mechanical properties		Refs.
			Synthetic	Charged	Cholesterol	Natural	Asymmetry	Protein <sup>d)</sup>	Residual additives	Characterization <sup>e)</sup>	
Swelling	Gel-assisted swelling	–	☒	☒	☒	☒	☐	☒	agarose/PVA	++	[61, 71, 73, 74, 129]
	Swelling glass beads	–	☒	☒	☐	☐	☐	☐	N/A	o	[77, 78, 130–134]
	Electroformation	–	☒	☒	☒	☒	☐	☒	N/A	+++	[61, 69, 135–141]
Emulsion	Inverted emulsion	+	☒	☒	☒	☒	☒	☒	not significant <sup>b)</sup>	+	[83, 89, 90, 116, 119, 120, 142–155]
	cDICE & eDICE	+	☒	☒	☒ <sup>b)</sup>	☒	☒ <sup>c)</sup>	☐	not significant <sup>b)</sup>	+	[88, 89, 101, 105, 106, 121, 156–159, 102–104, 108, 160, 161]
	Shaking method	+	☒	☒	☒	☒	☐	☒	not significant <sup>b)</sup>	+	[109, 162–165]
	DSCF	+	☒	☐	☒	☐	☐	☐	not reported	o	[110, 166]
	OLA	++	☒	☒	☒	☐	☐	☐	not significant <sup>b)</sup>	+	[91, 115, 126, 167]
	Surfactant-free microfluidics	++	☒	☒	☐	☒	☐	☐	not significant <sup>b)</sup>	+	[92, 168]

<sup>a)</sup> Size tuning and size monodispersity of GUVs for the different methods is categorized as follows: – (no size control whatsoever and/or large polydispersity), + (limited size control and/or relatively monodisperse GUV population), and ++ (full size control and/or GUVs highly monodisperse); <sup>b)</sup> See in-text description for further details; <sup>c)</sup> Formation of asymmetric GUVs has not been demonstrated using this technique, however, the method in principle allows for the sequential assembly of lipid monolayers; <sup>d)</sup> Membrane protein reconstitution shown during the GUV formation process; <sup>e)</sup> Extent of characterization of resulting GUVs produced by a certain method is expressed as follows: o (no characterization), + (limited amount of characterization studies; ≤2 publications), ++ (extensive characterization in literature; > 3 publications), and +++ (used as benchmark for clean membranes, extensive characterization in literature).

microfluidic assembly techniques (e.g., octanol-assisted liposome assembly (OLA),<sup>[91]</sup> other PDMS-based microfluidic methods<sup>[92–97]</sup> or hybrid methods;<sup>[84,98]</sup> reviewed in refs. [60, 99, 100]) which allow additional control over GUV size and in situ observation of GUV formation. In addition, several other techniques that offer enhanced control of the inverted emulsion process have been developed. One example is continuous droplet interface crossing encapsulation (cDICE),<sup>[88,101]</sup> and its recent adaptation termed emulsion cDICE (or eDICE)<sup>[102–104]</sup> which have been successfully implemented for actin cytoskeleton reconstitution experiments by various labs.<sup>[88,101,103–108]</sup> Other successful examples are the shaking method, which uses droplet-stabilized GUVs as intermediates,<sup>[109]</sup> and droplet-shooting centrifugal formation (DSCF), which makes use of a 3D-printed microcapillary.<sup>[110]</sup> While other techniques such as microfluidic jetting<sup>[111]</sup> have seen some success<sup>[112–114]</sup> they have not been widely adopted and further studies are needed to investigate the physicochemical properties of the resulting GUVs.

#### 4. Strengths and Limitations of Current Methods: A Comparative, State-of-the-Art Overview

As discussed in the previous section, there are two main approaches to forming GUVs. Well-studied swelling-based methods offer a wide range of lipid compositions, but at the expense of a limited encapsulation efficiency. Emulsion-based methods, on the other hand, excel at encapsulation but have only been shown effective with a limited set of membrane compositions and may entrap residual additives in the lipid bilayer. Since the ultimate artificial cell will need to comply with both membrane

and encapsulation requirements, there has been no one-method-fits-all solution so far. To compare the strengths and limitations of available GUV production methods, we review the state of the art of different methods and their compatibility with the specific membrane and encapsulation requirements outlined in Table 1. **Tables 2 and 3** provide an overview of the compatibility of different methods with membrane and encapsulation requirements, respectively. Note that we did not include natural swelling, swelling on paper (PAPYRUS) or textile, and jetting due to their limited use, characterization, or applicability, respectively. Our discussion on microfluidic-based techniques specifically focuses on two key publications that have shown significant advances for the construction of artificial cells: OLA, as published by Deshpande et al.,<sup>[91]</sup> and surfactant-free PDMS-based microfluidics, first published by Yandrapalli et al.<sup>[92]</sup> For a comprehensive overview of other microfluidic-based techniques, we refer the interested reader to other reviews.<sup>[60,99,100]</sup>

##### 4.1. Size

All methods discussed can generate GUVs within the preferred size range of 5–100 μm in diameter. However, considerable differences arise when considering size control and size monodispersity. Microfluidic-based techniques, like OLA, provide tight control over GUV size through channel design of the microfluidic chip and dynamic control of the used flow rates.<sup>[91,92,115]</sup> Consequently, GUV monodispersity is the highest in these methods and they are the only ones offering high-throughput production of same-sized GUVs. Non-microfluidic emulsion-based methods generally produce less monodisperse GUV populations.

**Table 3.** Overview of compatibility of different GUV production methods with encapsulation requirements based on state-of-the-art results.

	Method	Physiological buffers <sup>a)</sup>	Biological systems <sup>b)</sup>	Encapsulation efficiency <sup>c)</sup>	Cross-compatibility <sup>d)</sup>	Refs.
Swelling	Gel-assisted swelling	☒	Membrane-localized protein Filamentous protein	–	Low	[61, 71, 73, 74, 129]
	Swelling glass beads	☒	IVTT	–	Low	[77, 78, 130–134]
	Electroformation	☒	Membrane-localized protein Filamentous protein	–	Low	[61, 69, 135–141]
Emulsion	Inverted emulsion	☒ <sup>e)</sup>	Membrane-localized protein Filamentous protein IVTT Other	+	High	[83, 89, 90, 116, 119, 120, 142–155]
	cDICE and eDICE	☒ <sup>e)</sup>	Membrane-localized protein Filamentous protein IVTT Other	+	High	[88, 89, 101, 105, 106, 121, 156–159, 102–104, 108, 160, 161]
	Shaking method	☒ <sup>e)</sup>	Membrane-localized protein Filamentous protein Other	+	High	[109, 162–165]
	DSCF	☒ <sup>e)</sup>	IVTT	+	Low	[110, 166]
	OLA	☒ <sup>e)</sup>	Membrane-localized protein Filamentous protein	++	Low	[91, 115, 126, 167]
	Surfactant-free microfluidics	☒ <sup>e)</sup>	IVTT Other	++	Low	[92, 168]

<sup>a)</sup> Standard protein buffers such as Tris buffer, PBS, or HEPES buffer, or other buffers with physiological ionic strength ( $50\text{--}150 \times 10^{-3} \text{ M}$ ); <sup>b)</sup> Biological systems are divided into the following categories: peripheral membrane-localized protein, filamentous protein, IVTT systems, and other; <sup>c)</sup> Encapsulation efficiency for the different methods is categorized as follows: – (low encapsulation efficiency), + (strategies described to increase encapsulation efficiency), and ++ (encapsulation efficiency of  $\approx 100\%$ ); <sup>d)</sup> Cross-compatibility of different methods with different biological systems is categorized as low (number of different biological systems encapsulated  $\leq 2$ ) or high (number of different biological encapsulated systems  $> 3$ ); <sup>e)</sup> See in-text description for further details.

DSCF, the newest method we found, does not report any GUV size distributions,<sup>[110]</sup> but is thought to provide some selectiveness due to an inherent size filtration. For the inverted emulsion method and the shaking method using droplet-stabilized GUVs, no extensive size screenings and parameter studies have been reported to date, but recent studies gave first evidence that GUV sizes can be tuned to limited extent by changing the experimental conditions.<sup>[109,116]</sup> cDICE was originally presented as a promising method to allow for size tuning by controlling the capillary diameter used to generate the emulsion droplets.<sup>[101]</sup> However, larger capillary openings have since become the default to overcome issues related to capillary clogging, resulting in a complete loss of size control.<sup>[88]</sup> The more recent adaptation of cDICE, eDICE, refrains from using a capillary and results in similar GUV size distributions as cDICE.<sup>[104]</sup> This indicates an inherent size selectivity in these methods beyond that induced by droplet formation at a capillary orifice, predominantly yielding GUVs with a diameter around  $10 \mu\text{m}$ . By contrast, swelling-based methods typically result in an even more heterogeneous GUV size distribution.

While size control is useful for systematic screening, size polydispersity offers the advantage of screening size subpopulations within a single experiment, facilitating high throughput, provided that the GUV yield is sufficiently large.<sup>[78]</sup> Moreover, as experiments are often optically analyzed on a per-GUV basis, polydispersity in GUV size does not necessarily pose a disadvantage.

## 4.2. Compositional Complexity of the Lipid Bilayer

Swelling-based methods allow for reconstitution of membranes with a wide set of lipid compositions. Both gel-assisted swelling and electroformation have considerable advantages over the original natural swelling method, which is why they have largely replaced the latter in the field.<sup>[61]</sup> Both methods have been shown to be compatible with a wide range of lipids, including synthetic lipids, charged (both anionic and cationic) lipids, and natural lipids, and to also allow for the incorporation of relatively high molar ratios of cholesterol, as reviewed in Rideau et al.<sup>[61]</sup> Swelling-based methods, through swelling on heat-resistant surfaces, furthermore offer straightforward compatibility with elevated temperatures, making it possible to produce membranes with a wide set of phase transition temperatures, even including Archaeal lipid extracts with a melting temperature above  $80^\circ\text{C}$ .<sup>[117,118]</sup> Incorporation of natural lipids or cholesterol into GUV membranes made by swelling on glass beads has not yet been shown experimentally.

While charged membranes can be reconstituted with electroformation, the charge affects the GUV formation process, requiring careful fine-tuning of the formation parameters. Steinkühler et al.<sup>[46]</sup> found charged lipids to distribute asymmetrically in electroformed GUVs, an effect which could be countered by careful tuning of the voltage and temperature during electroformation. At ratios of  $> 50\%$  cholesterol, a demixing artefact occurred for electroformation, resulting in a lower final concentration of

cholesterol when compared to the initial ratio. Boban et al.<sup>[70]</sup> showed this effect could be reduced by tuning the lipid deposition method.

All emulsion-based methods, except DSCF, have, likewise, been found to be compatible with anionic lipids. Two emulsion-based methods, the shaking method and inverted emulsion, have even successfully constructed GUVs using *Escherichia coli* polar extract, providing a close mimic to complex biological membranes.<sup>[109,119]</sup> Both methods have also been shown to work with cationic lipids.<sup>[109,120]</sup> In principle, cholesterol can be incorporated up to nominal ratios of 20%–30% by the shaking method, inverted emulsion, and the OLA method, but the actual cholesterol content may differ from this input concentration; in case of cDICE, it was for instance shown that cholesterol ended up in a lower stoichiometric ratio than expected.<sup>[121]</sup> This issue could be overcome by delivering cholesterol to already-formed GUVs using cholesterol-loaded methylated  $\beta$  cyclodextrin molecules.<sup>[121]</sup> Another adaptation of cDICE, called double layer cDICE,<sup>[85]</sup> was introduced to overcome the cumbersome incorporation of cholesterol but has not led to any follow-up studies since. This sensitivity to cholesterol incorporation indicates a need for further clarification of the working principles of the emulsion-based GUV formation process.

While many methods have been shown to be compatible with different lipids, much less research has been conducted on the final stoichiometric ratios obtained in the lipid bilayer. As also illustrated by the examples above, a better understanding is needed of the extent to which different lipids and cholesterol are incorporated into the final membranes, so membrane functionality can be precisely tuned and controlled.

Obtaining an asymmetric bilayer composition requires sequential assembly of each lipid monolayer. Hence, asymmetry is inherently incompatible with swelling-based methods. Only inverted emulsion and eDICE offer the potential for obtaining a different lipid composition for inner and outer leaflet. Different groups have reported the assembly of asymmetric bilayers using inverted emulsion,<sup>[83,87,122]</sup> but this has yet to be demonstrated using eDICE. We would like to note that jetting, which we did not include here due to its limited compatibility with artificial cell research, does offer a neat way of producing asymmetric GUVs, as first shown by Richmond et al.<sup>[123]</sup> and later by Kamiya et al.<sup>[112]</sup>

Reconstitution of transmembrane proteins in GUVs has been extensively reviewed by Jørgensen et al.<sup>[124]</sup> In short, swelling-based methods generally allow for the direct incorporation of transmembrane proteins, yet protein dehydration is needed, which could potentially lead to denaturation in the process. Emulsion-based methods allow for transmembrane protein incorporation by solubilizing the protein in the oil phase, provided the protein is sufficiently soluble, but oil additives may be entrapped in the bilayer during incorporation. The shaking method is the only method providing a straightforward way of incorporating membrane proteins by making use of fusion of proteoliposomes during the GUV assembly process.<sup>[109]</sup> However, obtaining a controlled protein orientation is cumbersome, and generally, transmembrane protein reconstitution during GUV formation remains difficult. Several strategies exist for the incorporation of transmembrane proteins after GUV formation and encapsulation,<sup>[55]</sup> which are not further discussed here.

It is useful to note that the total amount of lipids necessary for various methods varies significantly, typically being lower for swelling-based methods, with, for example, only  $\approx 10 \mu\text{g}$  of lipids required for gel-assisted swelling compared to  $\approx 1.5 \text{ mg}$  for eDICE. The required quantity of lipids is particularly relevant when working with precious lipid samples, favoring swelling-based methods in those cases.

### 4.3. Mechanical Properties of the GUVs

To mimic drastic shape changes during growth or division, it is important to consider the mechanical properties of GUVs. One common concern with emulsion-based methods is the possibility of entrapment of residual amounts of additives into the lipid bilayer during solvent displacement, which could affect the chemical and mechanical properties of the formed lipid bilayers. Since swelling-based methods do not rely on solvent displacement, these methods are not affected by this issue. However, in contrast to emulsion-based methods, swelling-based methods, by relying on the hydration of lipid bilayers, can more easily give rise to unwanted multilamellar vesicles.<sup>[69,78]</sup> Furthermore, it has been shown that swelling on some polymer gels such as agarose or polyvinyl alcohol (PVA), can lead to membrane contamination from molecules released from the gel support, resulting in altered membrane properties (e.g., increased permeability, altered membrane interfacial tension, and lowered diffusion coefficients for lipids).<sup>[61,73,75,125]</sup>

For all emulsion-based methods except DSCF, several control experiments have confirmed the minimal effects of residual additives on membrane properties. All emulsion-based methods have been shown to result in unilamellar bilayers, confirmed by leakage assays incorporating alpha hemolysin.<sup>[88,91,92,101,109,116]</sup> Furthermore, for none of the resulting GUVs, there were optically detectable traces of any residues in the lipid bilayer.<sup>[88,91,92,101,109,116]</sup> Fluorescence recovery after photobleaching (FRAP) has been used to study lateral lipid diffusion coefficients, and it was shown that both the OLA method, surfactant-free microfluidics, and the shaking method result in GUVs with similar lipid diffusion coefficients to electroformed GUVs.<sup>[92,109,126]</sup> Fluctuation spectroscopy has also shown that cDICE-formed GUVs exhibit a comparable bending rigidity to electroformed GUVs, while for inverted emulsion, somewhat lower values were found albeit these changes were not statistically different.<sup>[101,116]</sup> GUVs produced using the shaking method have additionally been analyzed using cryotransmission electron microscopy (cryoTEM) and zeta-potential measurements, which confirmed no significant effects of any potential residual additives present in the lipid bilayer.<sup>[109]</sup>

Despite these extensive control experiments, a recent study by Faizi et al.<sup>[127]</sup> showed an altered shear surface viscosity for GUVs produced using gel-assisted swelling and inverted emulsion, attributable to gel remnants and residual oil, respectively. For experiments requiring perfectly clean membranes, it is therefore worth noting that solvent-free electroformation sets the benchmark for clean lipid bilayers.<sup>[56,128]</sup> Overall, many membrane studies and extensive characterization have been carried out for swelling-based methods, unlike emulsion-based methods, which have not been studied as thoroughly in this aspect.



#### 4.4. Encapsulation of Complex Solute Mixtures in Physiological Buffers

Encapsulation of macromolecules and essential small solutes in complex physiological buffers is indispensable for the reconstitution of a complex artificial cell. All discussed swelling and emulsion methods for GUV formation have been used with physiological buffers, often using standard protein buffers such as tris(hydroxymethyl)aminomethane (Tris) buffer, phosphate-buffered saline (PBS), or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer.

In emulsion-based methods, the outer aqueous solution is different from the inner aqueous solution but their osmolality needs to be matched, which is typically achieved by producing the GUVs in an outer aqueous solution supplemented with glucose. It is important to keep in mind that high concentrations of glucose can lower the pH of the outer solution, which in turn can have adverse effects on membrane properties. The lower pH is attributed to glucose's weak acidity resulting from the ability of its hydroxy groups to donate protons to water, in combination with the interconversion between the energetically favorable closed ( $\alpha$ -D-glucopyranose) and open/linear (D-glucose) forms.<sup>[169,170]</sup> Additionally, emulsion-based methods such as inverted emulsion, cDICE, eDICE, and DSCF, require a density difference between the GUV interior and the outer solution for successful GUV formation. This can be achieved by adding equimolar concentrations of sucrose and glucose in the inner and outer solution, respectively. While concentrations up to  $900 \times 10^{-3}$  M of sucrose/glucose have been used,<sup>[142]</sup> the effect of these high concentrations of sugar on protein and membrane properties have barely been studied.<sup>[171]</sup> As an alternative to using sugars, density gradient medium OptiPrep has been successfully used in cDICE and eDICE.<sup>[88,89,102–104,108,160,161]</sup> In the case of OLA, glycerol is required in both the inner and outer solution, along with the non-ionic triblock copolymer surfactant Pluronic P188 in the outer solution. It is important to note that these additional additives, especially at the high concentrations typically used, will affect protein and membrane properties during the experiments.

Inverted emulsion has been used for, among other systems, reconstitution of actin cortices,<sup>[90]</sup> encapsulation of tubulin,<sup>[143]</sup> in vitro transcription-translation (IVTT) systems,<sup>[119,144,146,150,152–154]</sup> RNA organelles,<sup>[142]</sup> and FtsZ filaments,<sup>[147,148]</sup> up to full in vitro reconstitution of the *E. coli* divisome machinery (including MinCDE, FtsZ, and FtsA).<sup>[149]</sup> Likewise, cDICE has been used to encapsulate a wide variety of systems. These include microtubules with kinesin motor clusters,<sup>[156]</sup> an actomyosin network coupled to the membrane<sup>[158]</sup> and contractile actomyosin rings.<sup>[105]</sup> Also the bacterial Min protein system,<sup>[89]</sup> fascin-actin bundles<sup>[159]</sup> and keratin networks<sup>[106]</sup> have been encapsulated using cDICE. Successful encapsulation of IVTT systems has also been demonstrated.<sup>[88]</sup> Furthermore, cDICE has successfully been used to encapsulate colloids, red blood cells,<sup>[101]</sup> small unilamellar vesicles (SUVs), DNA origami and even live bacteria.<sup>[88]</sup> Recently, there has been a clear shift from cDICE toward eDICE, implementing the proposed optimizations for cDICE presented in Van de Cauter et al.<sup>[88]</sup> eDICE has since been used to reconstitute actin cortices nucleated by the Arp2/3 complex<sup>[108]</sup> and VCA<sup>[104]</sup> and reconstitution of actomyosin networks.<sup>[103,159]</sup> Similar results have been obtained using the shaking method:

from encapsulation of F-actin with SUVs,<sup>[162]</sup> a DNA cytoskeleton mimicking actin rings,<sup>[165]</sup> and a DNA segregation module,<sup>[163]</sup> to cells.<sup>[109]</sup> DSCF has, so far, only been used to encapsulate IVTT systems.<sup>[110,166]</sup> Surfactant-free microfluidics showed encapsulation of a wide range of (biological) systems, from polystyrene beads to SUVs, IVTT systems<sup>[168]</sup> and even fibroblast cells.<sup>[92]</sup> Direct encapsulation of protein systems using this microfluidic method has yet to be reported. The OLA method has shown encapsulation of bacterial divisome proteins FtsZ and sZipA, including the colocalization of FtsZ filaments and ZipA at the membrane.<sup>[91]</sup>

Swelling-based methods, unlike emulsion-based methods, do not require any additives. An overview of encapsulation studies with electroformation and gel-assisted swelling can be found in Rideau et al.<sup>[61]</sup> Highlights are the successful reconstitution of actomyosin networks using gel-assisted swelling<sup>[72,74,172]</sup> and the reconstitution of an advanced protocell using electroformation, allowing light-controlled generation of ATP, in turn inducing the polymerization of actin.<sup>[173]</sup> Swelling on glass beads has proven particularly effective in encapsulating IVTT systems. Recent notable results include the de novo synthesis of MinD and MinE proteins,<sup>[132]</sup> the formation of FtsA-FtsZ ring-like structures yielding constricting GUVs,<sup>[130,131]</sup> the assembly of microtubules inside GUVs,<sup>[133]</sup> and DNA-programmed membrane synthesis<sup>[134]</sup> from IVTT systems. Despite these promising results with encapsulating IVTT systems, the method has not been used for encapsulation of other biological systems.

Generally, swelling-based methods are used more for membrane-oriented studies, while emulsion-based studies focus on studying the encapsulated content. This is in line with the favorable membrane properties of GUVs formed via swelling and the inherently superior encapsulation abilities of emulsion-based methods. For a more extensive recent overview of protein reconstitution in GUVs, we refer to Lopes dos Santos et al.<sup>[174]</sup> and Litschel et al.<sup>[155]</sup>

#### 4.5. Encapsulation Efficiency

To ensure a controlled protein concentration in the lumen of the GUVs and to achieve the correct protein stoichiometries, encapsulation efficiency is an essential factor to consider. Unfortunately, there is no generally accepted, standardized way of quantifying encapsulation efficiency, making it challenging to directly compare between different methods. Often, encapsulation efficiency is expressed qualitatively, by showing successful reconstitution of functional biological systems inside GUVs, instead of determining absolute luminal protein concentrations. First steps toward measuring absolute protein concentrations in GUVs have been taken by Supramaniam et al.,<sup>[175]</sup> who developed a microfluidics-based single-molecule approach, further emphasizing the need for quantitatively determining encapsulation efficiency.

Swelling-based methods are known to offer low encapsulation efficiency due to the incompatibility of the GUV formation mechanism with the encapsulation of large and charged molecules.<sup>[176]</sup> Tsai et al.<sup>[74]</sup> reported an encapsulation efficiency of about 50% for cytoskeletal actin-myosin networks, quantified by fluorescence intensity, using gel-assisted swelling. For swelling on glass

beads, the encapsulation efficiency of IVTT systems was shown to increase upon freeze-thaw cycles (also needed to break up multivesicular and multilamellar vesicles into GUVs).<sup>[78]</sup> The effect of content exchange between GUVs by repeated freeze-thaw cycles was also reported by Litschel et al.<sup>[89]</sup> A benefit of swelling on glass beads is that it is compatible with sample volumes as low as a few microliters,<sup>[77]</sup> which is convenient when working with precious samples.

Microfluidics-based methods offer the highest encapsulation efficiency as the injected solution gets directly encapsulated into GUVs. This has been shown for OLA and surfactant-free microfluidics. Yandrapalli et al.<sup>[92]</sup> reported a very high effective encapsulation efficiency of 95% for dispersible components (measured by fluorescence intensity), but a more variable encapsulation efficiency for large, solid objects like beads. No quantification has been reported for the shaking method while for DSCF an encapsulation efficiency of  $\approx 50\%$  for IVTT systems was reported by comparing fluorescence intensity to a bulk solution.<sup>[110]</sup> Encapsulation efficiency in cDICE was shown to be tunable by changing the composition of the lipid-in-oil dispersion, which alters the lipid adsorption rate.<sup>[88]</sup> The inclusion of a small fraction of PEG-ylated lipids likewise increased the encapsulation efficiency, which was shown by an increased protein expression by IVTT systems.<sup>[88]</sup> Loiseau et al.<sup>[158]</sup> noted that higher protein concentrations reduced the GUV yield for cDICE and the same effect was observed by Ganzinger et al.,<sup>[147]</sup> using inverted emulsion for GUV formation. Recently, Baldauf et al.,<sup>[104]</sup> using eDICE, observed a supersaturation effect (up to 1.7 $\times$  the nominal concentration) for encapsulation of higher actin concentrations, demonstrating that even emulsion-based methods give a polydispersity in encapsulation efficiency. It is important to note that for many emulsion-based methods, the detailed GUV formation mechanisms are still unknown. It is, therefore, also unknown how, and to what extent, different inner solutions and protein concentrations influence the GUV formation process.

#### 4.6. Cross-Compatibility of GUV Production Methods and Different Biological Systems

Encapsulating the different modules needed for an artificial cell within a single GUV, arguably poses the biggest hurdle to date. Different GUV production methods show varying degrees of success in encapsulating different biological systems (i.e., peripheral membrane-localized protein, polymerizing protein, IVTT systems, and other). The above review clearly demonstrates strengths and limitations in terms of the different methods regarding the types of biological systems that can be encapsulated effectively. Highly specialized methods like swelling on glass beads have been found to be reliable for encapsulating IVTT systems, but may not be as effective for encapsulating other biological systems. In contrast, cDICE and its improved variant eDICE have shown compatibility with encapsulating a wide variety of biological systems.<sup>[88,103,104]</sup>

Achieving successful GUV formation under the conditions needed for a given biological system of interest requires a thorough understanding of the chemical and physical properties of each system, as well as their impact on GUV formation and the encapsulation process. However, few studies have been con-

ducted on the effects of different protein and protein systems on GUV formation mechanisms. Further research is needed to fully elucidate the underlying mechanisms and develop more effective strategies for making GUV production work seamlessly with different biological systems.

#### 4.7. Operational Requirements

Thus far, we have exclusively discussed the requirements for the produced GUVs themselves. However, an additional and often undervalued factor to consider is the “workability” of the production method. Considerations include the accessibility of the method to potential new users, the adaptability of the method to new experimental conditions, or the vastness of the parameter space, all of which have not yet been extensively covered in the literature for any of the methods discussed.<sup>[61,69]</sup> We introduce these criteria here under the umbrella term “operational requirements”. It may be argued that such operational requirements carry little significance if the primary objective is to show that a well-defined final goal (i.e., the formation of a complex artificial cell) can be achieved. Nonetheless, we would like to stress the importance of operational requirements for methods to become established within the field, be adopted by new research groups, and enhance collaboration between research groups. The current abundance of diverging protocols for each GUV formation method poses obstacles for reproducibility and suggests a lack of robustness for most methods as constant adaptations are required to apply a method to a new experimental system. Hence, a balance needs to be struck between working toward scientific advancements with a certain method and ensuring the method works reproducibly and robustly.

As a general rule, swelling-based methods are simple to set up, require minimal equipment and do not require extensive training of the experimentalist. As outlined above, both gel-assisted swelling and swelling on glass beads have not proven their applicability in an equally wide range of experimental conditions when compared to electroformation. Electroformation excels under a wide variety of conditions, both regarding membrane composition and buffer conditions. While electroformation is, therefore, sometimes claimed to be applicable in virtually any condition, it needs to be acknowledged that these results were achieved with an equally wide range of protocols, often despite similar conditions,<sup>[61]</sup> potentially related to the lack of understanding of the process of GUV formation. It is unclear to what extent this disparity in protocols results in GUVs with different physicochemical properties. The parameters affecting GUV formation in electroformation include the electroformation chamber (a.o. electrode materials, electrode cleaning protocol, dimensions), the lipid deposition method, electrical field parameters, temperature, and total duration of GUV formation. As many groups use homemade electroformation chambers, there is considerable variation in protocols. This makes it difficult to standardize protocols, even for similar conditions, resulting in a lack in translational reproducibility. To address this issue, there are commercially available devices such as the Vesicle Prep Pro, which has already been used for a small number of recent studies.<sup>[137–139,177]</sup> Exploring the parameter space of electroformation experiments given by the method itself thus remains a time-intensive endeavor, yet

important, as lipid oxidation (which occurs at different voltages for different lipid species),<sup>[178]</sup> duration, and temperature are all important factors to consider upfront.

Compared to swelling-based methods, relatively little information is available for the operational requirements of emulsion-based methods. Emulsion-based methods like inverted emulsion, the shaking method, or cDICE/eDICE only require simple laboratory setups and are therefore relatively accessible to new users. Microfluidic-based techniques, on the other hand, often have a high entry point due to their reliance on advanced fabrication techniques like soft lithography and the need for specialized devices. The adoption of these techniques typically requires additional resources, expertise, and investments compared to other emulsion-based and swelling-based methods, further illustrated by the limited number of follow-up studies for many microfluidic-based methods proposed. Recent studies have focused on mapping the input parameters of emulsion-based methods and highlighted its day-to-day variability,<sup>[88]</sup> with the role of factors such as humidity and lipid-in-oil solution preparation yet to be fully investigated.

In summary, the operational requirements for both swelling- and emulsion-based methods remain an area of active research, with significant efforts underway to address the associated challenges. While for swelling-based methods, particularly electroformation, the focus is on countering the divergence of protocols, the primary aim for emulsion-based methods is on elucidating the vast parameter space. There are ongoing efforts in the scientific community that aim to address the reproducibility gaps in these methods by an open-science approach.

## 5. Moving Forward: A Blueprint for Advancing GUV Formation for Artificial Cell Research

The creation of an artificial cell has been a hotly tackled challenge over the past decade and, while significant progress has been made, the artificial cell is still far from being a reality. In this review, we explored the potential of GUVs as a suitable artificial cell container, needed for building an artificial cell from the bottom-up. To assemble different modules of cell function inside a single GUV, the GUVs and GUV production methods should comply with a list of requirements including obtaining biologically relevant membrane properties and controlled encapsulation, and further ideally meet operational requirements such as reproducibility and ease of use. Here, we provided an overview of the current state of the art in GUV production methods. Despite the availability of more than 10 commonly used methods, none of them fully meets all the necessary requirements for the artificial cell at this moment. Most promising results (i.e., challenging multi-component reconstitution experiments) have been achieved using emulsion-based methods, yet exactly for these methods characterization studies remain limited.

On the road ahead, we propose efforts should be intensified to elucidate physical principles behind the different GUV formation methods to provide a basis for knowledge-based optimization and adaptation. Swelling-based methods are relatively well-characterized, but emulsion-based methods, despite their widespread use in the field, still lag behind, likely mainly due to their relatively short history. For these methods, it is important to further evaluate the effects of residual additives in the lipid

bilayer and the effect of density-increasing chemicals to ensure optimal mechanical properties. Since lipid composition is crucial for controlling mechanical and biological functionality of the membrane, it is important to know its exact composition. Some initial work has been done on comparing the ratio of input lipids to the final ratio obtained in the GUV bilayer, yet a detailed understanding of what determines the final lipid composition of the GUV produced, is still missing. Comparative qualitative and quantitative analysis of the GUVs produced by different methods is likewise important. While there are competing priorities and these studies are often time-consuming, they can provide a solid foundation for future progress by establishing standardized protocols and by providing appropriate metrics for assessing and comparing GUVs and the method used to provide them.

In addition, our focus should be on convergence rather than expansion. The ultimate goal of creating an artificial cell should serve as the driving force, with resources directed toward encouraging challenging integration experiments. Promoting open science, for example by including comprehensive methods sections and full protocols in published papers, will encourage exchange and collaboration between different labs, and help move toward a more systematic and collaborative approach to GUV formation and ultimately the creation of an artificial cell. Collaboration between different research groups, sharing of data and resources, and open access to publications and protocols can accelerate progress and avoid duplication of effort. This way, we can continuously push the limits in our efforts to mimic the essence of life and move one step closer to turning the artificial cell into a reality, ultimately recreating life in the lab.

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## Conflict of Interest

The authors declare no conflict of interest.

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