Reconstitution of actin-based cellular processes: Why encapsulation changes the rules
Fabina Binth Kandiyoth, Alphée Michelot

To cite this version:
10.1016/j.ejcb.2023.151368. hal-04304096

HAL Id: hal-04304096
https://hal.science/hal-04304096
Submitted on 1 Mar 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License
Reconstitution of actin-based cellular processes: Why encapsulation changes the rules

Fabina Binth Kandiyoth, Alphée Michelot *

Aix Marseille Univ, CNRS, IBDM, Turing Centre for Living Systems, Marseille, France

ARTICLE INFO

Keywords:
Cytoskeleton
Actin
Biomimetism
Encapsulation
Polymerization
Recycling

ABSTRACT

While in vitro reconstitution of cellular processes is progressing rapidly, the encapsulation of biomimetic systems to reproduce the cellular environment is a major challenge. Here we review the difficulties, using reconstitution of processes dependent on actin polymerization as an example. Some of the problems are purely technical, due to the need for engineering strategies to encapsulate concentrated solutions in micrometer-sized compartments. However, other significant issues arise from the reduction of experimental volumes, which alters the chemical evolution of these non-equilibrium systems. Important parameters to consider for successful reconstitutions are the amount of each component, their consumption and renewal rates to guarantee their continuous availability.

1. Overview of current efforts to reconstitute actin polymerization-dependent cellular processes

For researchers of the past and future decades, reconstituting the molecular processes of the cell is an essential challenge to understand the subtleties of the mechanisms involved, and for creating new tools inspired by nature (Ganzinger and Schwille, 2019; Vale, 2014). These efforts lead to a gradual increase in the complexity of the biomimetic systems that are developed as our knowledge of the cell advances. The many molecular processes dependent on actin polymerization are no exception to this rule (Pollard, 2003). These processes include all those where the free energy is consumed by the actin polymerization reaction, but exclude, for example, those where a majority of the energy is consumed by myosins. A review of the community’s efforts reveals that while some of these processes are relatively well reconstituted, others have received much less attention (Lopes dos Santos and Campillo, 2022). For example, our capacity to mimic actin-based propulsion mechanisms, such as those used in cell migration, is much more advanced than our capacity to reproduce actin-based pulling mechanisms, such as those found in endocytosis where actin is involved in membrane invagination. The reasons for these unequal progresses are certainly due to our better knowledge of some biological processes than others, but also to the different levels of complexity and engineering required for these different reconstitutions.

Another striking observation is that many successful reconstitutions are carried out under experimental conditions where neither the size nor the geometry of the system is comparable to the cellular environment. In vitro experiments are often performed between slides and coverslips, which are small samples at the human scale but remain virtually infinitely large at the scale of an eukaryotic cell. In this review, focused on this issue, we will discuss all the scientific challenges of working in confined environment and the difficulties that emerge when trying to scale experiments down to systems of the size of a cell. By (an abusive) analogy to thermodynamics, we will confront what we call “open” systems, where molecular exchanges are largely possible between the site of assembly and the rest of the sample, to “closed” systems, where experimental efforts are made to encapsulate biological material inside a compartment more comparable to the environment of the cell, and where no exchange of material is possible with outside. As an order of magnitude of the volume difference between these experimental configurations, open systems correspond to experiments performed with a few microliters of solution. By comparison, closed systems should have volumes comparable to those of eukaryotic cells, which, with a few exceptions, range from 0.01 to 1000 pl. Thus, there is a 3-to-8-orders of magnitude difference in volume between open and closed configurations.

In the following sections, we will examine the main differences between the two configurations, and why a simple encapsulation of a functional open system does not usually lead to a successful reconstitution in a closed environment.

* Corresponding author.
E-mail address: alphee.michelot@univ-amu.fr (A. Michelot).
2. Reconstitutions of force-generating actin networks in open systems

2.1. Early attempts to reconstitute actin-based motility in vitro

For obvious reasons of experimental simplicity, the development of biomimetic systems based on actin polymerization was performed first using open configurations. Initial attempts correspond to the reconstitution of Arp2/3 complex-based motility at the surface of bacterial pathogens such as *Listeria monocytogenes*. These bacteria, which naturally invade host cells by assembling a propulsive tail of polymerizing actin filaments, can form similar actin structures and become motile when placed in cytoplasmic extracts (Marchand et al., 1995; Theriot et al., 1994; Welch et al., 1997). This experimental system was gradually evolved towards more artificial conditions, when bacteria were replaced by spherical micrometer-sized beads coated with various Arp2/3 complex nucleation promoting factors (Bernheim-Grosawasser et al., 2002; Cameron et al., 1999). As an alternative to rigid beads, actin has also been assembled around deformable objects to assess the force field applied by the actin network (Boukellial et al., 2004; Giardini et al., 2003; Helfer et al., 2000; Upadhyaya et al., 2003). An important step was later made when all the proteins necessary for this system to function were identified, and that cellular extracts could be replaced by a minimal number of purified proteins (Loisel et al., 1999). From the cell to the most artificial systems, rates of actin assembly and motility are conserved and on the order of several \( \mu \text{m} \text{min}^{-1} \). If these studies provide biochemical insight into how actin polymerization is controlled in cellular processes such as bacterial propulsion or lamellipodial protrusion, these experiments are generally important for our understanding of all cellular processes where sustained actin polymerization provides force (Allard et al., 2021). Thus, motility assays remain to this day key model experiments for many types of reconstitutions of actin-based cellular processes. In parallel, feedback between experiments and various types of mathematical and computational models has enabled us to understand the underlying physical principles in detail (Achard et al., 2010; Boujemaa-Paterski et al., 2017; Dayel et al., 2009; Kawso et al., 2012; Manhart et al., 2019; Paluch et al., 2006; Zhu and Mogilner, 2012).

One of the main difficulties to overcome in these experiments is to find conditions in which actin polymerization remains efficient over long periods of time, usually up to one to several hours. Spontaneously, purified actin placed in physiological buffer conditions assembles into filaments, and only a critical concentration \( c_c \) of about 0.1 \( \mu \text{M} \) remains as actin monomers (Drenckhahn and Pollard, 1986). Thus, at steady-state, a solution of actin filaments “treadmills” at a slow rate corresponding to \( k_{\text{treadmill}} \). This treadmilling rate of about 1 subunit per second corresponds to an elongation of approximately 0.2 \( \mu \text{m} \text{min}^{-1} \), which is very slow compared to the fast polymerization reactions observed in cells (Fritzsche et al., 2013; Lai et al., 2008; Theriot and Mitchison, 1991). Thus, biomimetic systems require additional actin regulators whose function is to increase the fraction of polymerizable actin, in order to accelerate the growth of the actin filaments up to cellular rates (Carlier and Fantaloni, 2010).

2.2. “F-actin” versus “G-actin” assays

Two strategies are generally employed experimentally to achieve this goal. The first strategy is to prepare a pre-polymerized actin source and then shift the equilibrium of the system towards the production of actin monomers. These so-called “F-actin assays” (F stands for filamentous) typically rely on two additional actin regulators: ADF/cofilin which catalyzes the disassembly of aging actin filaments and promotes the production of new actin monomers; and capping protein, which blocks the vast majority of the actin filament barbed ends present in solution to prevent the rapid consumption of the monomers (Loisel et al., 1999; Michelot et al., 2007; Romero et al., 2004). The second strategy consists in starting from a stock solution of actin monomers, previously prepared in a buffer where the polymerization reaction is inhibited, and then to place these monomers in polymerization conditions but in the presence of regulatory proteins which will inhibit the polymerization reaction. These so-called “G-actin assays” (G stands for globular) typically rely on two additional actin regulators: profilin, which inhibits the spontaneous nucleation of actin into filaments and blocks pointed end elongation; and capping protein, as a complementary mechanism to block the polymerization of the filaments forming spontaneously in solution (Achard et al., 2010; Akin and Mullins, 2008; Reynmam et al., 2011).

The two F- and G-actin strategies are different in that the vast majority of actin in solution is found in different states, either filamentous or globular. However, both strategies are based on the principle of increasing the concentration of polymerizable actin, in order to funnel these extra monomers efficiently to sites where active actin polymerization is desired. Theoretically, experiments performed with similar concentrations of actin, profilin, ADF/cofilin and capping protein but different actin sources should reach similar steady-states and be equivalent; however, in practice, because steady-states are reached within several hours, these systems remain globally out of equilibrium for the duration of these experiments. Neither strategy is better than the other, but the design of the experiment is usually chosen according to the scientific question that is being investigated. For example, an experimentalist seeking to determine the effect of an F-actin regulatory protein should favor an assay performed from a G-actin source, because the concentration of this F-actin binding protein will remain stable and known. Using an F-actin assay, a fraction of the regulatory protein of interest would necessarily bind to the filamentous actin present in bulk and its free concentration would not be controlled.

3. Methods of encapsulation and their (dis)advantages

The encapsulation of purified actin within closed cell-sized microenvironments has been successfully performed, with liposomes, emulsion droplets and microwells being the most commonly used methods (Mulla et al., 2018) (Fig. 1). Early attempts to integrate actin and actin regulators into confined systems used a sub-class of liposomes called giant unilamellar vesicles (GUVs). These spherical vesicles have a diameter varying from a few micrometers to several tens of micrometers, which corresponds to volumes in the femtoliter to picoliter range and close to the volume of eukaryotic cells (Has and Sunthar, 2020). An advantage of GUVs is that they feature a deformable membrane interface comparable to the cell membrane, and membrane tension can be controlled by adjusting the osmolarity of the media inside and outside the vesicles. Controlling osmotic pressure also enables to provide excess membrane surface by emptying the vesicles, allowing a larger phase space of deformation to be explored. Initially, established methods such as electroswelling were used to form the GUVs (Honda et al., 1999; Limozin and Sackmann, 2002), but these methods lead to vesicles with a broad distribution of sizes and only a few of the vesicles produced are unilamellar. Moreover, as the vesicles are formed in the medium containing the proteins, it is difficult to use this method to encapsulate high concentrations of proteins specifically inside the vesicles. To overcome these difficulties, subsequent encapsulation experiments have used various inverse emulsion techniques, in which liquid droplets are first placed in a lipid-in-oil solution to form a lipid monolayer on the droplet surface, then transported through a lipid-water interface to form a second lipid layer around the forming vesicles (Pautot et al., 2002). These techniques require controlled displacement of the vesicles, which can be achieved by jetting or centrifugation (Abkarian et al., 2011; Pontani et al., 2009; Stachowiak et al., 2009; Van De Cauter et al., 2021). The possibility of forming domains of specific lipid compositions by liquid phase separations offers the potential for local recruitment of proteins of interest.
if a molecule has a maximum efficiency at a concentration corresponding to the presence of 1000 molecules in a volume V, the reduction of the system to a volume V/2 will imply that on average, 500 molecules are still present and their collective effect remains comparable. However, if we consider the extreme case where only one molecule would be present on average in the volume V, the division of this volume in V/2 implies that this molecule becomes either present or absent, which can greatly affect the behavior of the system if this molecule has an important function. For the size range covered by eukaryotic cells, these effects are usually minor but not always negligible for the least expressed proteins (Fig. 2a). For example, a haploid budding yeast cell has a volume within the range of 10–100 μm³, 70% of which is not occupied by organelles (Uchida et al., 2011). In these cells, if a protein concentration of 1 μM corresponds approximately to 4200–42,000 molecules, a protein concentration of 1 nM corresponds only to 4–42 molecules, the temporal evolution of which is more difficult to model using a standard continuous description.

4.2. Consequences of system volume reduction on reagent availability

Even if all the most important molecules of the system are present in sufficient numbers and that stochastic effects can be neglected, additional scaling issues arise during encapsulation due to the size reduction and change in geometry of the system.

Encapsulation has for consequence to change the available volume of reagents without changing in similar proportions the size of the reconstituted product (Fig. 2b). As a result, the amount of reagents required for the assembly of the structure does not change, while the amount of reagent available decreases by a significant amount (i.e. by the cubic root of the characteristic dimension of the system). Typically, in an open system, the dimension of the sample is large enough so that the local assembly of the process affects the concentrations of reactants moderately, as if the reservoir was infinite. When the reaction volume gradually decreases by several orders of magnitude, this infinite reservoir approximation no longer applies. As a result, the reservoir state changes rapidly, with pronounced depletion effects for the most limiting reactants (Fig. 2b). As all the reagents are not necessarily depleted in the same proportions, this phenomenon also leads to stoichiometric changes in the concentrations of the different reagents. The decrease in concentration of reagents will in turn cause the chemical reactions involved in the assembly of the biomimetic system to slow down and eventually stall.

Fig. 1. Different platforms of encapsulation with some of their advantages and disadvantages.
4.3. Consequences of adapting the size and geometry of the reconstituted process on reagent consumption

An analogous problem can arise if the size or spatial organization of the reconstituted structure is governed by the geometry of the compartment. For example, a process that cannot be reconstituted locally, but rather over the entire inner surface of the compartment, adds an additional difficulty to the experiment. The amount of reagent needed to assemble this structure is proportional to the square root of the characteristic dimension of the system and potentially important compared to the limited size of the reservoir. If this difficulty is not anticipated, there is a risk for the depletion effects mentioned above to be amplified.

5. Reconstitutions of actin network assembly in closed systems

5.1. Formation of organized actin networks in closed systems

The development of encapsulation protocols has paved the way for more complex experiments, starting with simple actin polymerization reactions under confined conditions (Lopes dos Santos and Campillo, 2022). These approaches have opened up new biophysical questions, such as the influence of the cell cytoskeleton on membrane fluctuations, deformations or mechanical properties (Guevorkian et al., 2015; Honda et al., 1999; Limozin et al., 2005), or reciprocally, the influence of shape or size limitation on the spatial organization of cytoskeletal polymers. Actin filaments persistence length (characteristic length defining polymer rigidity), which is around 18 µm (Gittes et al., 1993; Isambert et al., 1995), is comparable to the size of the encapsulation devices described earlier. As a result, actin filaments are spatially constrained and their diffusion properties are altered, leading to their spatial reorganization into bundles and asters at protein concentrations well below those reported in open configurations (Bashirzadeh et al., 2021; Deshpande and Pfohl, 2015; Limozin and Sackmann, 2002). In spherical geometries, these bundles can arrange circularly and form structures resembling cytokinetic rings, particularly in the presence of crosslinkers (Baldauf et al., 2022; Gaessens et al., 2006; Litschel et al., 2021; Miyazaki et al., 2015). These systems were rapidly complexified, integrating new regulatory proteins to control the spatial organization of actin networks. While bead motility is one of the most standard assays to reconstitute actin networks assembly in open systems, cortical actin network reconstitution is one of the most frequently developed for closed systems. In vivo, these networks are formed of filamentous actin beneath the plasma membrane. They provide rigidity to the cell and play the central role in a wide range of cellular processes like migration, division and adhesion (Salbreux et al., 2012). The actin cortex is highly dynamic, undergoing continuous assembly and disassembly of the filaments (Biro et al., 2013; Fritzsche et al., 2013). Experimentally, a simplified actin cortex can be reconstituted at the interface of a lipid vesicle by addressing a nucleation-promoting factor to the membrane; in the presence of actin and of the Arp2/3 complex, a branched actin network assembles spherically at the membrane (Baldauf et al., 2023; Dürre et al., 2018; Guevorkian et al., 2015; Murrell et al., 2011; Pontani et al., 2009; Wubshet et al., 2023).

Fig. 2. Effects of confinement on the evolution of chemical reactions. (a) Average number of molecules found in a reaction volume as a function of their concentration. (b) Schematic comparison of two reconstitutions using open or closed configurations, with orders of magnitude of typical availability volumes and surfaces of assembly. In an open configuration (top panels), accumulation of molecules (in green) at the reaction site (typically a micrometer-sized bead, in red) leads to a negligible depletion in bulk (left panels). As a consequence, all the molecules of interest are maintained at similar concentrations (right panel). In a closed configuration (bottom panels), accumulation of molecules (in green) at the reaction site (typically the inner surface of a GUV, in red) can lead to its depletion in bulk (left panels). Limiting reagents are depleted more rapidly, which leads to changes in stoichiometric ratios.
5.2. Depletion effects lead to the formation of non-dynamic networks in assembly conditions

The comparison between a bead-based assay in an open configuration and the reconstitution of a cortex in a closed configuration is interesting because both experiments aim to form identical networks while presenting a geometric inversion. The depletion effects mentioned above can be estimated for each system respectively. A simple calculation shows that the concentration of monomeric actin decreases mono-exponentially:

\[ G = G_0 e^{-k_{a,b} [BE]} \]

with a characteristic time \( \tau = \frac{1}{k_{a,b} [BE]} \).

In these formulas, \( G_0 \) is the concentration of actin monomers at initial time points, \( k_{a,b} \) is the association constant of actin monomers at barbed ends mentioned earlier and \([BE]\) is the concentration of barbed ends in the system. \([BE]\) changes during the experiment as the filaments are nucleated and capped, but we can approximate its value in this simple calculation by referring to the order of magnitude of free barbed end density \( d \approx 100 \mu m^{-2} \) estimated for this type of network from experiments (Achard et al., 2010; Akin and Mullins, 2008; Bieling et al., 2016; Wiesner et al., 2003), models (Dayel et al., 2009; Kawksa et al., 2012; Wang and Carlsson, 2015) or in cells (Carlsson, 2016; Holz and Vavylonis, 2018; Mueller et al., 2017; Vinzenz et al., 2012).

\[ [BE] = \frac{S d}{N_a V} \]

where \( S \) is the surface covered by network growth, \( N_a \) is the Avogadro number and \( V \) is the available reagent volume.

A numerical application then clearly shows that depletion effects are considerable for volumes corresponding to closed conditions. For example, the assembly of a branched network on a 1 \( \mu m \)-diameter bead present at a density of 10,000 beads/\( \mu l \) (typical of an experiment in an open configuration) results in the depletion of the monomeric actin reservoir in a characteristic time of about 334 min. By comparison, assembly of the same network inside a 10 \( \mu m \)-diameter compartment (typical of an experiment in a closed configuration) results in depletion of the monomeric actin reservoir in a characteristic time of only 8 s.

These depletion effects have been known for a long time and have been the subject of several experimental demonstrations in the area of cytoskeletal research (Fig. 3). A striking example in cells is the size of mitotic spindles, which scales with cytoplasmic volumes during embryogenesis in amphibians, invertebrates and mammals (Courtois et al., 2012; Greenan et al., 2010; Hara and Kimura, 2013; Wühr et al., 2008). Reconstitutions of spindle assembly in liquid droplets of different sizes and shapes demonstrate that compartment volume is the key parameter controlling spindle size (Good et al., 2013; Hazel et al., 2013) by limiting the amount of biological material (Fig. 3 - top right). This example highlights how protein limitation and depletion effects are not necessarily a threat for cells, but can serve as efficient and essential mechanisms for controlling reaction rates and size of organelles. Similarly, the actin monomers available for the polymerization reaction are also present in limited quantities in cells. This property is highlighted by the fact that the different actin networks of the cell compete for this limited actin reservoir, a phenomenon known as actin network homeostasis (Fig. 3 - bottom) (Burke et al., 2014; Suarez and Kovar, 2016). Each actin network does not control its size independently, but as a function of the availability of polymerizable actin and the polymerization rates of all the networks (Antkowiak et al., 2019; Carlier and Shekhar, 2017; Rotty et al., 2015; Suarez et al., 2015; Wubshet et al., 2021).

Similarly, all in vitro experiments seeking to reconstitute actin network assembly in cell-size compartments face these depletion effects, because of the high concentration of actin filament barbed ends required for efficient network growth. Consequently, experiments based solely on

![Fig. 3. Importance of reagent availability and depletion effects in cell biology. This schematic represents a cell assembling two networks (in blue and orange) from a common pool of reagents. The size of the networks is not determined independently, but depends on the availability of key reagents and how they are shared between the two assembly pathways. As a result, increasing cell volume (at constant reagent concentration - top right) increases the size of the reservoir which leads to larger networks. Improving recycling mechanisms (top left) for the most limiting reagents also lead to the growth of networks dependent on these reagents. Inhibition of a specific pathway (bottom) reduces the size of a network to the benefit of other networks which can benefit from the limiting reagents.](image)
actin assembly in closed systems systematically lead to the formation of non-dynamic structures, which can be revealed by FRAP experiments (Bleicher et al., 2020) or by the progressive slowing down of the polymerization reaction (Colin et al., 2023). To see the effect of the polymerization reaction in real time, experimental tricks need to be considered, such as integrating pores in the membranes of liposomes in order to trigger actin polymerization directly under the microscope by addition of salt and ATP (Limozin and Sackmann, 2002; Pontani et al., 2009).

5.3. Fast recycling of actin networks is critical to maintaining continuous polymerization in closed systems

All of the experiments described previously have successfully created cell-like microenvironments with actin assembly machineries to polymerize purified actin, providing methods for studying the properties of filamentous networks at steady state. However, what has been mostly overlooked in these systems, and requires careful consideration, is the dynamic nature of actin networks in vivo. This rapid dynamic is essential to maintain continuous actin polymerization, which generates forces. Moreover, as the size of the networks is governed by their assembly and disassembly rates, controlling these dynamics ensures that actin networks maintain an optimal size (Fig. 3 - top left).

In vitro reconstitution of actin-dependent cellular processes therefore requires the identification of experimental conditions under which actin networks undergo continuous restructuring at cellular rates. The aim is to reach actin network renewal rates similar to those measured in non-muscle cells, which are known to be rapid, with characteristic turnover times of the order of seconds to minutes (Fritzsche et al., 2013; Lai et al., 2008; Theriot and Mitchison, 1991). Additional proteins need to be introduced into the system to accelerate the kinetically limiting steps of the actin cycle, which are the disassembly of older filaments and their recycling into polymerizable monomers (Pollard et al., 2000). This recycling machinery must also ensure that all the system’s most limiting components, which are the ones most likely to be depleted first, are recycled efficiently. For this reason, experimental protocols include in particular ATP regenerating systems, essential for supplying the energy required to maintain actin network dynamics.

Because of the difficulty of identifying the molecular set required to maintain these rapid dynamics, reconstituted systems based on the encapsulation of cellular extracts give so far the most convincing evidence of the possibility to reach fast actin turnover in vitro. For example, continuous actin cortex turnover (Abu Shah and Keren, 2014) and contractile acto-miosin network flows (Ierushalmi et al., 2020; Malik-Garbi et al., 2019) are observed convincingly when encapsulating Xenopus egg extracts in water-in-oil emulsions. Cell extracts are powerful experimental models for the identification of critical components and their function through their depletion or addition as purified proteins (Michelot et al., 2013; Michelot and Drubin, 2014). These “top-down” approaches do not, however, ensure that all the critical factors contributing to the phenomenon of interest have been identified. A key element in this progress is likely to be our better understanding of the factors which, in addition to ADF/cofilin and profilin, enable more rapid disassembly and recycling of actin networks (Gressin et al., 2015; Hao et al., 2008; Jansen et al., 2015; Kotla et al., 2019; Nadkarni and Brieher, 2014; Shekhar et al., 2019). Cyclase-associated protein (CAP) is certainly one of these factors, as it enables continuous actin assembly at the surface of microbeads in chambers of around 140 pl, which compares with the cytoplasmic volume of the largest eukaryotic cells (Colin et al., 2023).

6. Concluding words

We argue that the process of encapsulating a biomimetic system that is functional in an open configuration goes far beyond technical aspects. Its success also depends on a good adaptation of the experimental conditions to a new geometry and spatial scale. The path to success necessarily involves a good understanding of the biochemical reactions involved, the activity of the proteins involved, the molecular mechanisms of the cell, and the chemical problems associated with the limited availability of components. In parallel, mathematical and computational modeling approaches are invaluable in guiding towards good experimental conditions.

Although this review is written in the context of actin polymerization-dependent processes, most of the principles described here are also valid for the reconstitution of other cellular processes. Overall, these efforts should lead to a better understanding of how the dynamics of non-equilibrium molecular systems are maintained.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alphée Michelot reports financial support was provided by Fondation pour la Recherche Médicale.

Acknowledgments

The authors acknowledge Clément Campillo, Etienne Loiseau, Antoine Jégou and the two anonymous Reviewers for their careful reading of the manuscript and helpful comments.

References


