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Aspects of Biological Replication and Evolution Independent of the Central Dogma: Insights from Protein-Free Vesicular Transformations and Protein-Mediated Membrane Remodeling

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Abstract

Biological membrane remodeling is central to living systems. In spite of serving as "containers" of whole-living systems and functioning as dynamic compartments within living systems, biological membranes still find a "blue collar" treatment compared to the "white collar" nucleic acids and proteins in biology. This may be attributable to the fact that scientific literature on biological membrane remodeling is only 50 years old compared to ~ 150 years of literature on proteins and a little less than 100 years on nucleic acids. However, recently, evidence for symbiotic origins of eukaryotic cells from data only on biological membranes was reported. This, coupled with appreciation of reproducible amphiphilic self-assemblies in aqueous environments (mimicking replication), has already initiated discussions on origins of life beyond nucleic acids and proteins. This work presents a comprehensive compilation and meta-analyses of data on self-assembly and vesicular transformations in biological membranes—starting from model membranes to establishment of Influenza Hemagglutinin-mediated membrane fusion as a prototypical remodeling system to a thorough comparison between enveloped mammalian viruses and cellular vesicles. We show that viral membrane fusion proteins, in addition to obeying "stoichiometry-driven protein folding", have tighter compositional constraints on their amino acid occurrences than general-structured proteins, regardless of type/class. From the perspective of vesicular assemblies and biological membrane remodeling (with and without proteins) we find that cellular vesicles are quite different from viruses. Finally, we propose that in addition to pre-existing thermodynamic frameworks, kinetic considerations in de novo formation of metastable membrane structures with available "third-party" constituents (including proteins) were not only crucial for origins of life but also continue to offer morphological replication and/or functional mechanisms in modern life forms, independent of the central dogma.

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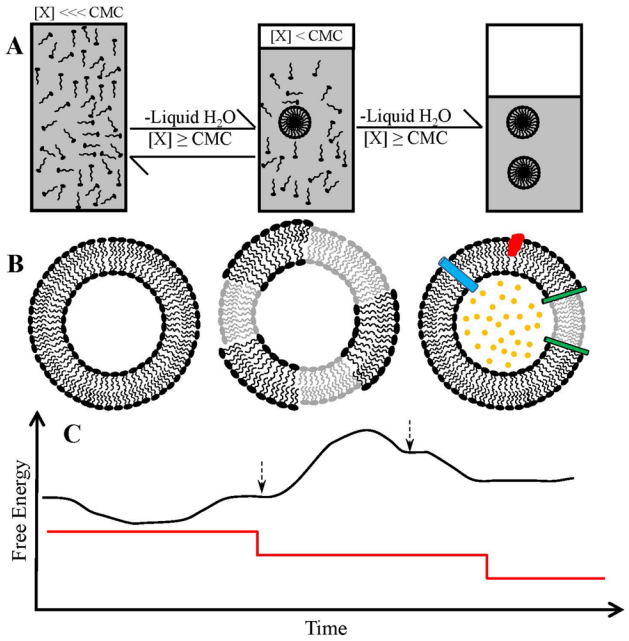
Graphic Abstract



Keywords Vesicles · Membrane fusion · Influenza · Virus · Liposomes · Origin of life

Introduction

The discovery of enzymes as cellular constituents was a major landmark in the history of science (Kühne 1877). Prior to this discovery, appreciation of "small" single cells as living units, having the ability to appear de novo, was also coupled with the belief that only living cells (called *ferments*) had a "magical" ability to carry out a variety of chemical transformations at ambient conditions. This "magic" was a signature of life. The fact that all enzymes were proteins (believed for more than a century after first use of the word "enzyme" by Kühne) immediately put the onus of biological replication (and evolution of living systems since the origins of life) on proteins. These were molecules found in all living cells and, now known to be responsible for all biochemical reactions. More than half-a-century later, this onus shifted to DNA subsequent to remarkable work of Griffith (1928), Oswald et al. (1944), Chargaff (1950), Hershey and Chase (1952), Rosalind Franklin, and Maurice Wilkins, that led to often called "discovery-of-the-century", i.e., structure of DNA by Watson and Crick (1953). Almost another half-a-century later, discovery of catalytic functions of RNA by Altman and Cech (Kruger et al. 1982; Guerrier-Takada et al. 1983) initiated discussions on an RNA-world hypothesis for origins of life (Cech 2012). In a span of ~ 100 years covered above, views on molecular basis of origins of life, biological replication, and evolution of biological systems shifted from proteins to DNA to possibly DNA and RNA. Emerging from this time span, functional manifestations of proteins now define "phenotypes" and nucleic acids are considered as indicators of biological relatedness in experimental as well as evolutionary time scales (Woese et al. 1990). All this while, biological membranes (specifically their lipid constituents), in spite of being the "third front" in living systems, i.e., beyond nucleic acids and proteins, continue to remain as comparatively passive components in origins of life and biological evolution (Bansal and Mittal 2015). In fact, a common "textbook" visualization of cell membranes comes from Singer and Nicolson (1972)—the fluid-mosaic model in which a mosaic of non-phospholipid entities (e.g., integral proteins) is embedded in a phospholipid bilayer acting as two-dimensional viscous fluid. More than a decade later, a phenomenological thermodynamic perspective with a purely mechanical emphasis resulted in a mattress model (Mouritsen and Bloom 1984)—a cell membrane was visualized as an elastic mattress of lipids with non-lipid impurities (e.g., proteins) embedded as localized springs (with distinct elasticities) in the mattress. While neither of the above models focused on heterogeneous details (e.g., local curvatures), they allowed visualization of "average" cell membranes through specific organizational frameworks; the former providing some features relevant to nano-scales while laying foundations for the well-appreciated asymmetry in cell membranes. Over the years, these models have transformed

into a more mosaic view of cell membranes by incorporating localized, often transient, interactions (including those involving peripheral membrane and cytoskeletal proteins along with components of extracellular matrix) resulting in distributions of heterogeneous domains/rafts with different relative mobilities (Jacobson et al. 1995; Nicolson 2013; Almeida 2014; Nicolson and Ferreira de Mattos 2021). However, the above visualizations of cell membranes, while increasingly attributing active roles to their lipid components, still do not consider those lipids in contexts of origins of life and biological replication (Mittal et al. 2020).

Some of the earliest work on development of model membrane systems (Bangham and Horne 1964; Bangham et al. 1965), transitioning into development of liposomes (Bangham 1972), indicated close similarities to behavior of biological membranes—on the level of whole cells as well as intracellular organelles. In fact, the early excitement of being able to replicate both biological structures and their behavior in absence of proteins and nucleic acids is well captured by the following representative statement from Bangham and Horne (1964): *Certainly the lesions produced in the model membrane of lecithin–cholesterol are commensurate with the damage obtained in biological cells when either saponin or lysolecithin is used as a lytic agent*. In spite of the above and a sustained interest in the possibilities of creating "simple" protocells and life-like properties from "non-living" materials (Rasmussen et al. 2004; Mittal 2009), biological replication as a "property" still resides only in the domain of the central dogma. In this context, it needs to be appreciated that literature on experimental kinetics and thermodynamics of membrane remodeling, starting from protein-free systems to protein-mediated transitions/transformations, is only ~ 50 years old. In contrast, there are over 100 years of literature on proteins and a little less than 100 years of literature on nucleic acids. Therefore, it is not surprising that exploration of possible biological replication mechanism(s) offered by self-assembling lipid systems in aqueous environments, free from nucleic acids and proteins, is very recent (Mittal et al. 2020; Steinkühler et al. 2020, 2021). However, proper analyses of scientific literature on remodeling in self-assembled membranes and/or vesicular transformations opens up possibilities of "phenotypic" and/or "functional" replication mechanisms in nucleic acid- and protein-free aqueous environments. Further, transitional events recorded during protein-mediated membrane remodeling show availability of kinetic windows where thermodynamic constraints may become available for (re)creation of structures and/or functions in protein-free systems. In this regard, one of the most biologically relevant and ubiquitous membrane remodeling events is membrane fusion. From the variety of structural and transformational aspects involved in fusion of bilayers, to kinetic and thermodynamic considerations during morphological transitions, membrane fusion covers almost all "variables" pertaining to membrane remodeling.

In this work, we comprehensively analyze literature on assembly and transformations in biological membranes, starting from protein-free vesicular transformations and culminating in protein-mediated membrane remodeling, with a focus on membrane fusion. To every extent possible, we have collected data from original experimental literature (that has withstood the test of time and scrutiny) instead of relying on specific interpretations of that data presented in numerous reviews. While highlighting and appreciating some landmark experimental results on kinetics and thermodynamics of protein-free vesicular systems, we discuss the emergence/establishment of Influenza Hemagglutinin (HA, also spelled as Haemagglutinin) as a prototypical membrane fusion protein. In fact, HA is arguably as important for understanding molecular-level functioning in biology from the perspective of ubiquitous membrane remodeling dynamics crucial for life, as have been enzyme systems from the perspective of the protein folding problem. We discuss how HA-mediated membrane fusion systems have attained significance in biology similar to that of enzymes that provided "functional assays" (e.g., ribonuclease and chymotrypsin) and were responsible for opening the gates of current understanding on structure and function of proteins (Anfinsen 1972). Next, we make a comprehensive comparison of various biological vesicular systems—enveloped mammalian viruses, extracellular, and intracellular vesicles, especially based on size and certain physico–chemical properties in context of biological membrane remodeling. Finally, we lay the foundations of development of a theory on how kinetic transitions in membranes may be "stabilized" by proteins toward formation of dynamic, yet metastable, structures away from equilibrium configurations preferred by the same membrane constituents in aqueous systems. Such structures play integral roles in living systems and understanding their formation is crucial toward gaining insights into origins of life, biological self-assembly, and replication.

Protein-Free Vesicular Assemblies, Transformations, and Membrane Remodeling

Table 1 compiles sizes of vesicular assemblies (and transformations) formed (and reported) in some pivotal experimental studies with specific lipid compositions in aqueous systems. Each of these studies represent significant methodological and/or analytical advances, including experimental assays and their interpretations. While the pre-1975 studies mostly relied on freeze fracture and electron microscopy techniques for visualization of membranes assemblies and vesicular transformations, phase behaviors of lipid mixtures were

Table 1 Protein-free vesicles

Dia (nm)	Constituents	Primary observation(s)	Reference(s)
22, 100–1000	Lecithin, Ovolecithin, Lysolecithin, Chol, K-phosphotungstate	Membranous formations—rings with helical structures, rod-like micelles, vesicles, MLVs, bilayers with varying thickness	Bangham and Horne (1964)
> 1000	Ovolecithin:Chol:dicetyl-Phosphoric acid (75:10:15)	Uni- and multi-lamellar assemblies, "functional" implications pertaining to membrane permeability	Bangham et al. (1965)
> 1000	DMPC, DPPC, DSPC, DPPE	Melting temperature (Tm)-dependent phase separation—So and Lα phases in binary mixtures	Shimshick and McConnell (1973)
~ 200, ~ 460, 100–300, 200–500, 200–1000	DPPC, PC, PG, PS, Chol	LUVs and oligolamellar vesicles with large internal aqueous volumes encapsulating variety of water-soluble molecules with high efficiencies	Szoka and Papahadjopoulos (1978)
100–1000	PC:PA (98:2)	Proton-hydroxyl permeability measurements	Nichols et al. (1980)
22–25, 100	PS	Ca^{2+}-induced aggregation and fusion of SUVs and LUVs, content mixing assay	Wilschut et al. (1980)
~ 230	Egg yolk PC, Octyl glucoside (detergent)	Unilamellar vesicles formation on removal of detergent from mixtures	Cohen et al. (1981)
15	PS	Na^{+}-induced aggregation of SUVs, distinction in rates and extent of aggregation (equilibrium)	Bentz and Nir (1981a, b)
~ 25, ~ 100	PS	Ca^{2+}-induced aggregation and leaky fusion of SUVs/LUVs, kinetic characterization	Nir et al. (1982), Bentz et al. (1983b)
~ 30, ~ 100	PS	Aggregation kinetics, bilayer destabilization, fusion rates induced by Ba^{2+}, Ca^{2+}, Sr^{2+}, Mg^{2+}	Bentz et al. (1983a)
100–1000	Egg yolk lecithin, detergent ($C_{12}E_8$)	Effects of detergent removal kinetics on assembly of unilamellar vesicles and their permeability	Ueno et al. (1984)
> 2000	Asolectin, Ergosterol	Leakage during membrane fusion, adhesive forces promote membrane tension and rupture	Niles and Cohen (1987)
10–50	DMPC, DPPC	Thermodynamically spontaneous fusion of SUVs to form intermediate-sized unilamellar vesicles	Lentz et al. (1987)
200, 100–1000	DOPC, DOPE, *N*-Methylated DOPE	LUVs, MLVs, SUVs, Lα to H_{II} phase transitions in membrane fusion (with inverted micelle intermediates, interlamellar attachments)	Ellens et al. (1989)
> 1000	PC (lecithin), DPPC, β-arachidonoyl γ-palmitoyl-PC	Fast (< 2 min) assembly of GUVs, "Cell-sized" proteoliposomal assembly	Moscho et al. (1996)
> 1000	DOPC, Chol	Estimation of line tension, effects of differently shaped inclusion molecules in membranes	Karatekin et al. (2003)
> 1000	DOPC, SM, Chol	Lo/Ld phase separations, morphologically distinct assemblies	Baumgart et al. (2003)
> 1000	O-ethyl-PC, DOPC, DOPG (glycerol)	Vesicle fusion by video fluorescence microscopy	Lei and MacDonald (2003)
> 1000	DOPC, DPPC, POPC, Chol (Binary/Ternary mixtures)	Phase diagrams, temperature-dependent domain ripening and curvatures	Veatch et al. (2004)
> 1000	DOPC, POPC, palmito-SM, Chol	Miscibility phase diagrams, coexistence of Lo/Lα, and formation of So at different temperatures	Veatch and Keller (2005)
> 1000	DOPC, DPPC, Sterols	Sterol-specific assemblies with and without coexisting phases (So/Liquid)	Beattie et al. (2005)

Table 1 (continued)

Dia (nm)	Constituents	Primary observation(s)	Reference(s)
200–300	Bacterial—PE	Liposomal assemblies from bacterial lipids	Gupta et al. (2008)
50, 100–400	"Prebiotic" mixtures of decanoic (capric), lauric, oleic, and octanoic acids	Glycerol monoacyl amphiphiles stabilize "assemblies"; leaky "vesicles," mixing of "membranes" at "high" temperatures	Maurer et al. (2009)
200–400	DOPE:DOPC:Chol	Critical "Compartmentalization" Concentration of stoichiometrically defined compositions	Mittal and Grover (2010)
150–200	CTAB, Chol, DOPC	Vesicle preparations using a compressed fluid-based method are more homogeneous	Elizondo et al. (2012)
> 1 µm	DOPC:DOPG (8:2)	"Electroformation" of vesicles, transient asymmetry and morphological transitions	Steinkühler et al. (2018)
> 1 µm	l-α-PC (Soy), DOPE, Bacterial—CL, PE, PG	Ca^{2+}-dependent vesicle and "subcompartment" formation from surfaces by interfacial events	Spustova et al. (2021)

Lipids→*Chol* Cholestrol, *CL* Cardiolipin, *CTAB* Cetrimonium bromide, *DM* Dimyristoyl, *DO* Dioleoyl, *DP* Dipalmitoyl, *DS* Distearoyl, *PA* Phosphatidic acid, *PC* Phosphatidylcholine, *PE* Phosphatidylethanolamine, *PG* Phosphatidylglycerol, *PS* Phosphatidylserine, *SM* Sphingomyelin, Others→*Dia* Diameter, Phases (H_{II} Hexagonal, *Lα* Liquid crystalline, *Lo* Liquid ordered, *Ld* Liquid disordered, *So* Solid), *Tm* Melting temperature, *GUV* Giant unilamellar vesicle, *LUV* Large Unilamellar vesicle, *SUV* Small unilamellar vesicle, *MLV* Multilamellar vesicle

quantitated using differential scanning calorimetry or spectra of spin labels distributions that depended on membrane phases. By 1980, protein-free systems had developed enough to be able to provide interesting insights into "functional" aspects of biological membranes, such as permeability, in addition to visible mimicking of compartmental morphologies observed in cells. Subsequent to refinements in methods of liposomal preparations, 1980 to 1990 was a remarkable decade resulting in development of crucial membrane fusion assays for varying liposome sizes (size represents curvature, especially in smaller assemblies). Contents mixing assays using Tb^{3+}/dipicolinic acid, carboxyfluorescein, drug molecules, enzymatic substrates, and a variety of different sized molecules transferring between fusing compartments were developed. In addition, lipid mixing assays (not listed in Table 1), namely—dequenching of the self-quenched R18 (Octadecyl Rhodamine B) upon lipid mixing (Struck et al. 1981), FRET between *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)—Rhodamine subsequent to lipid mixing between bilayers separately labeled with NBD-PE and N-Rh-PE (Hoekstra et al. 1984) and the Pyrene excimer assay (Stegmann et al. 1993) were also established. The Pyrene assay overcame some of the limitations of the earlier lipid mixing assays such as non-specific probe transfer on close apposition of bilayers. A key achievement in this period was the development of rigorous kinetic frameworks (e.g., Bentz et al. 1983a, 1983b) and thermodynamic constraints (e.g., Lentz et al. 1987) required to properly interpret data on vesicular preparations and their utilization in membrane remodeling experiments. Analytical tools clearly extracting distinct kinetics of aggregation, lipid mixing, contents mixing, and final extents of measurements (fusion) from the data were developed. Specifically noted was the fact that neither were vesicular assemblies at thermodynamic equilibrium, nor did the end points of assays depict thermodynamic end points of membrane remodeling. These rigorous quantitative treatments of experimental data on fusion of protein-free lipid bilayers (and later protein-mediated membrane fusion) laid the foundations for a variety of subsequent studies on protein-mediated remodeling of biological membranes directly or indirectly, including the Nobel-accorded (in 2013) efforts on *discoveries of machinery regulating vesicle traffic, a major transport system in our cells*. (Malhotra et al. 1988; Perin et al. 1990; Sollner et al. 1993; Hata et al. 1993; Weber et al. 1998).

By early 1990s, developments in (and more accessibility of) fluorescence microscopy (epifluorescence followed by confocal) allowed direct visualization of vesicular assemblies, phase separations, and dynamics of membrane remodeling. Thus, as is evident from Table 1, substantial contributions emerged out of work on preparation and visualization of GUVs. This allowed experimental verification, and further development, of concepts such as line

tension, mean/Gaussian curvatures, bending rigidity, and tilt/splay in lipids that are predominantly theoretical (but physically important). Start of the new century brought progress toward quantitative visualization of transitions and distributions of different phases in individual membrane assemblies compared to populations of assemblies. Important phase diagrams of defined amphipathic mixtures self-assembling into membranes in aqueous environments continue to emerge out of such progress till date. Interestingly, the last couple of entries in Table 1 represent a somewhat cyclical nature of scientific exploration—(i) the work of Steinkühler et al. (2018) is reminiscent of the seminal work of Chernomordik et al. (1987) that investigated different stages of electric field-driven morphological transitions and curvature formations in planar bilayers and (ii) the work of Spustova et al. (2021) is reminiscent of the seminal work of Wilschut et al. (1980), Nir et al. (1982), and Bentz et al. (1983b); spontaneous vesiculation and "subcompartment" formation on removing Ca^{2+} observed recently may be simply interpretable as the dominance of reverse rate constants in the older studies that used Ca^{2+} as an inducer for creation of larger assemblies (fusion products) from smaller vesicles. Nevertheless, the recent studies allow a much better appreciation of the original literature from 1980s, especially for interpreting experimental results on protein-free vesicular assemblies, their transformations, and overall membrane remodeling in the context of origins of life and structural replication mechanisms (of biological compartments) independent of the central dogma.

In addition to the above, some very recent methodological advances provide fascinating ways to address a key limitation of previous vesicular assemblies. Unlike cell membranes, liposomal membranes are devoid of membrane asymmetry. Bhatia et al. (2018) overcame this limitation by inducing (transient) asymmetry in GUV bilayers by utilizing small amounts of glycolipids (ganglioside) GM1 whose local concentrations in respective monolayers changed due to desorption of GM1 from the outer monolayer as a result of extravesicular "dilution" by preparation buffer. Subsequently, Bhatia et al. (2020) induced (transient) asymmetry in osmotically balanced GUVs with encapsulation of a sugar different from the extravesicular sugar in the suspension solution, thereby varying local hydration environments of the inner and outer monolayers. Such innovative approaches resulting in remarkable protein-free vesicular assemblies and transformations are gradually pushing "in vitro" systems closer to mimicking previously "unmatched" features of living cells ("in vivo" systems).

From Advent of Liposomes to Development of Experimental Assays for Membrane Fusion

Figure 1 shows a timeline from the advent of liposomes to development of experimental assays for membrane fusion; initially in protein-free and later applied to protein-mediated membrane fusion experiments. By early 1970s, model membrane systems had been developed from a variety of amphipathic mixtures in different aqueous environments (Bangham 1972). While experimental work on "functional" manifestations (e.g., permeability, rupture/repair) of these model membranes continued, theoretical frameworks and mechanistic models involving physico–chemical properties of amphipathic molecules in aqueous systems emerged. Today, conceptual formulations of (i) the hydrophobic effect (Tanford 1973; Tanford 1978; see legend to Fig. 1) and, (ii) shape parameters for amphipathic molecular constituents (Israelachvili et al. 1976) of assemblies in aqueous environments (e.g., micelles, bilayers), have the same significance in understanding of biological membranes as secondary structures are significant in understanding of protein structures. In fact, such is the remarkable validity and applicability of a theoretical concept such as shape parameter that it is found

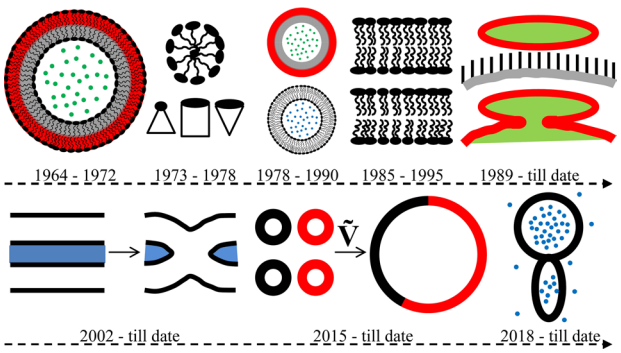


Fig. 1 Timeline from advent of liposomes to development of experimental systems and assays for membrane assemblies and fusion (see text for details). The timeline captures some of the key original experimental advances—from the first liposomes in the 1960s, to the latest protein-free vesicular assemblies involving "electroformation" of GUVs from heterogenous SUVs (Bhatia et al. 2015) along with inducing of transient bilayer asymmetries by varying intra- and extravesicular conditions (Bhatia et al. 2018, 2020). Here, it is pertinent to enforce the importance of properly interpreting the hydrophobic effect—the term "hydrophobic interaction" neither implies any force nor is it a real "interaction"—it is actually exclusion by water. In absence of an aqueous environment, "hydrophobic" molecular entities do not show any tendency to interact with each other. Thus, while the word "hydrophobic" may appear to imply phobia from water, experimentally and scientifically it represents only exclusion from/by liquid water (anecdotally interpretable as water's phobia for such molecules/entities rather than the other way around). The figure is drawn only for illustration purposes and not to any scale

to be a molecular signature of bilayer constituents even in simulations of flat bilayers (Bansal and Mittal 2013).

The 1980s also saw complementation of membrane fusion between curved membranes by visualization of flat planar bilayers spontaneously formed by defined compositions in aqueous environments with the help of Teflon and similar materials serving as substrates/surfaces. Chernomordik et al. (1987) utilized/developed one such experimental assembly for recording structural transitions during membrane fusion between two planar bilayers while coupling their experimental findings with a theoretical stalk model (Kozlov and Markin 1983). In fact, on one hand, the work of Wilschut et al. (1980), Bentz and Nir (1981a, b) created the foundations for combining theoretical and experimental kinetics in membrane fusion. On the other hand, Chernomordik et al. (1987), Ellens et al. (1989) and Siegel (2008) created the foundations for combining theoretical and experimental thermodynamics of remodeling during membrane fusion. Next, as introduced earlier, developments in fluorescence microscopy allowed direct and visual measurements of membrane fusion. In this direction, Blumenthal, Zimmerberg and colleagues, utilized erythrocytes [red blood cells (RBCs)] and RBC "ghosts" fusing with HA-expressing cells for direct monitoring of events occurring during biological membrane fusion (Morris et al. 1989; Sarkar et al. 1989; Herrmann et al. 1993). This was possible since glycophorins on RBCs are heavily sialated and sialates serve as receptors for HA. The above was made possible by development of the HA-expressing cell—RBC ghost experimental system by Doxsey et al. (1985) and Sambrook et al. (1985). With this, the era of studies on membrane fusion of protein-free lipid bilayers transitioned to predominantly protein-mediated membrane fusion. The basic fundamentals of experimental assays pioneered during that time continue to serve till date, with liposomes (especially SUVs representing curvature-related parameters in bilayers, and LUVs) and protein-free lipid systems ("lipid nanosystems") shifting in relevance as drug delivery vehicles in subsequent times (Meers 2022).

In relation to the above, it is pertinent to note that later applications involving X-ray (diffraction, scattering) and/or neutron scattering have allowed a high-resolution structural characterization of protein-free lipid bilayers further validating and/or confirming earlier findings. While predominantly using flat model membranes, these experiments have resulted in quantitative estimates for bilayer interaction forces (Wong et al. 1999a, b) along with properties such as membrane thickness, degree of heterogeneity, tilt, bending moduli and phase separations etc. (Karmakar and Raghunathan 2005; Kamal and Raghunathan 2012; Tristam-Nagle 2015; Nagle 2017). Specific high-resolution visualizations of spatial organizations and/or local curvatures and/or suitability of membrane systems for (or resistant to) remodeling continue to complement/confirm earlier results obtained from relatively low-resolution assays and observations (see lower left panels "2002-till date" in Fig. 1). For example, on one hand Yang and Huang (2002, 2003) were able to directly visualize a "static" stalk formation in dehydrating lipid membrane stacks. On the other hand, Salditt and colleagues captured transient "dynamic" stalk formations, presenting them as structural fusion assays, in aligned multi-lamellar stacks of protein-free-lipid membranes (Aeffner et al. 2012; Scheu et al. 2021).

Influenza HA-Mediated Membrane Fusion: Establishment of a Prototypical Fusion System

In the late 1960s, first reports pertaining to internalization of several viruses (Type 2 Parainfluenza virus, Influenza virus, New Castle Disease virus, Herpes Simplex virus, and Sendai virus) by different cells came from light microscopy (Howe et al. 1967) and electron microscopy (Howe et al. 1967; Morgan and Howe 1968 and references there in). These studies did indicate events such as "binding," "fusion," "penetration," and "uncoating" of virus particles with different cells—based on protocols involving incubation of virus particles at 4 °C for "binding" and raising the temperature to 37 °C for "penetration" into cells. Using a similar protocol and electron microscopy, Haywood (1974) reported "fusion" and "penetration" of Sendai virus into ganglioside containing model membranes, presumably to avoid cell culture-related artifacts. However, artifacts pertaining to sample preparation and/or image acquisition in all the above experiments were still difficult to segregate. It is interesting to note that Sendai virus, known to induce syncytia in cell cultures, does not require low pH for membrane fusion. Influenza virus did not induce similar syncytia but was shown to "penetrate" target membranes in the above studies. Clearly something was amiss in experimental protocols. To this end, Helenius et al. (1980) followed by White and Helenius (1980) reported low pH-driven membrane fusion by the Semliki Forest virus (SFV), using assays involving cells and liposomes, but free of possible artifacts in the earlier studies. The fusion assays were "end-point" and comparatively robust. Catalytic activity of enzyme trapped in liposomes on viral RNA subsequent to fusion and inhibition of viral infectivity in cells treated with lysosomotropic agents (that increased lysosomal pH) were indirect assays compared to direct kinetic assays (Table 1). However, the experiments were ground-breaking for discovering the need for low pH "trigger" required for SFV membrane fusion. The aftermath was unequivocal demonstration of low pH-induced membrane fusion by Influenza and the Vesicular Stomatitis viruses—the experimental assay was formation of cell syncytia (White et al. 1981).

Figure 2 shows the timeline for establishment of Influenza HA as a prototypical membrane fusion protein (note that the figure also includes crucial pre-influenza HA experiments also for completeness). Ectodomain of HA was the first membrane fusion protein whose crystal structure was solved (Wilson et al. 1981). The solved structure was that of bromelain-cleaved HA at neutral pH and the remaining decade (plus some more years) went into consistent efforts to decipher low pH conformation(s) of HA. A key development was creation of HA-expressing fibroblast cell lines (Doxsey et al. 1985; Sambrook et al. 1985) that allowed development of HA-mediated cell–cell membrane fusion systems triggered by low pH. This resulted in some exemplary experiments monitoring double-labeled RBC ghosts fusing with HA-expressing cells when exposed to low pH. Experimental protocols developed for model membrane fusion systems were now directly applied to HA-mediated fusion. Convenient size of the cell–cell fusion system allowed real-time monitoring of fusion events using patch clamp, lipid mixing, and contents mixing assays. Influential discoveries on sequence of events, from flickering pores allowing ion transfer, to lipid mixing, to opening, and expansion of fusion pores during protein-mediated membrane fusion, were made (Sarkar et al. 1989; Spruce et al. 1989; Tse et al. 1993; Zimmerberg et al. 1994; Leikina et al. 2004). These were accompanied by periodic experiments of Influenza virus fusing with liposomes (e.g., Stegmann et al. 1995). However some key questions remained: what was(were) the exact role(s) of HA in fusion? What was the architecture of the fusion site? These questions were reflective of almost a century-old investigation on how proteins work as enzymes (see "Introduction" section).

To this end, Ellens et al. (1990) conducted one of the most seminal experiments, which introduced experimental transformation of fundamental concepts of three-dimensional chemical reaction stoichiometry from solutions to lesser dimensions (in this case surface reactions). Monitoring fusion of fibroblasts expressing different surface densities of HA to same target membranes was equivalent to laboratory titrations aimed at uncovering chemical reaction stoichiometries in solutions. And the results were astounding—HA surface density was not linearly related to the extents of fusion observed. Ellens et al. (1990) unambiguously showed involvement of more than one HA in fusing membranes and allowed development of the first estimates of the architecture of a protein-mediated membrane fusion site (Bentz et al. 1990). However, a more accurate estimation of the fusion site architecture required kinetic data in addition to extents. Further, assignment of specific roles to individual HA molecules in the fusion site required knowledge of low pH structure of HA. In regards to the latter, a remarkable computationally driven prediction by Carr and Kim (1993) was confirmed by solving of low pH crystal structure of HA, called TBHA2 (shown at 1994 in Fig. 2, explained in figure legend). The structure not only showed one of the most dramatic conformational changes discovered in any protein till that date, but also provided possible mechanisms for membrane fusion along with an experimentally verifiable concept of "metastable" states in general protein folding. This was followed by a series of experiments that generated invaluable kinetic data on HA-mediated membrane fusion with a variety of experimental systems—first fusion pore formation in cell-planar bilayer fusion (Melikyan et al. 1995), virus-liposome fusion at different pH values, with different strains of Influenza with different target membranes (Shangguan et al. 1996), and cell–cell fusion (Danieli et al. 1996; Blumenthal et al. 1996). Kinetic data generated above was comprehensive since (a) it used the same standardized

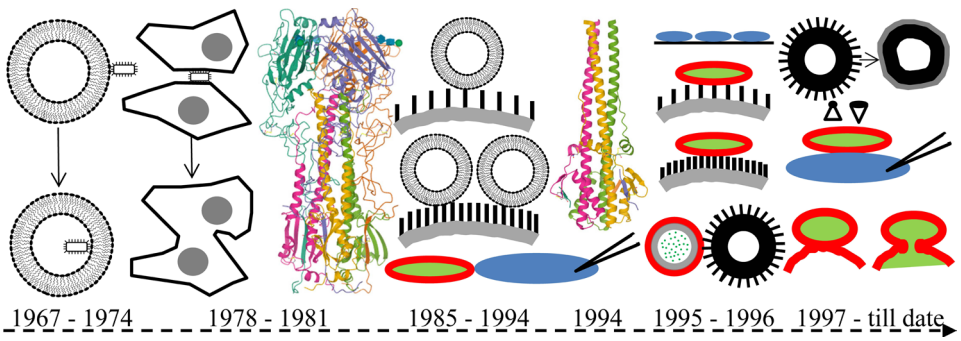


Fig. 2 Timeline for establishment of Influenza HA as a prototypical membrane fusion protein and development of experimental assays for studying HA-mediated membrane fusion (see text for details). Wilson et al. (1981) solved the structure of bromelain-cleaved HA (called BHA) at neutral pH—the first structure shown is from PDB (Berman et al. 2000) PDB ID: 5HMG (Wilson et al. 1981; Weis et al. 1990); the second structure, called TBHA2, shown is PDB ID: 1HTM (Bullough et al. 1994) which is trypsin + thermolysin + bromelain—cleaved HA at low pH. Overall interpretation from 5HMG and 1HTM is that HA is a trimer with each monomer having two subunits—HA1 responsible for receptor binding and HA2 responsible for membrane fusion. The figure is drawn only for illustration purposes and not to any scale

HA-expressing cell lines or quantitatively well-characterized Influenza virus particles and (b) it came from diverse, but equally important methods, for monitoring different steps of membrane remodeling during fusion till that date, i.e., electrophysiological measurements, lipid mixing, and contents mixing assays. Interestingly, individual interpretations of the data did not appear to converge on the same (or even similar) architecture of the fusion site - differences attributable to different measurements (single-first-fusion-pore-event vs single-cell–cell-fusion-membrane-conductivity vs single-cell–cell–lipid/content mixing vs population-cell–cell–lipid-mixing vs population-virus–liposome–lipid/content mixing).

Remarkably, a mass-action model initially developed by Bentz (2000a), extended by Mittal and Bentz (2001), followed by Mittal et al. (2002b), was able to extract an unambiguous consensus architecture of the HA-mediated membrane fusion site from the above comprehensive kinetic datasets (Bentz and Mittal 2003). Specific roles were assigned to receptor-bound HA and free HA at the fusion site while incorporating cryo-EM based data of Shangguan et al. (1998) on low pH induced inactivation (i.e., fusion incompetence) of influenza virions. The emergent "textbook" picture defined a minimal aggregate size (ω = minimum number of HA molecules aggregated to form a fusion site) and a minimal fusion unit (n = minimum HA molecules required to undergo the dramatic conformational changes observed from BHA to TBHA2 for creation of the first fusion pore). Based on the data, receptor-bound HA molecules were not a part of the "n" (minimal fusion unit) and assisted in creation of restricted fusion site that eventually expands. The significance of this mass-action model to HA-mediated membrane remodeling during fusion is arguably akin to that of Michaelis–Menten kinetics in enzyme function—with an additional advantage of the primary parameters in the fusion model, i.e., ω and n, being mechanistic (in contrast to V_{Max} and K_{M} which are phenomenological). While the above was focused on arriving at the architecture of HA-mediated membrane fusion site from the perspective of HA molecules, Chernomordik et al. (1997, 1998) carefully dissected lipidic intermediates formed during HA-mediated merger of bilayers using cell–cell fusion assays—with fascinating approaches that involved arresting or promoting membrane curvature-based remodeling during HA-mediated fusion by addition of exogenous lipids with different shape parameters to the experimental system. By combining electrophysiological measurements, lipid, and contents mixing assays, Chernomordik and colleagues were able to create "textbook" visualizations for protein-mediated membrane remodeling events during HA-mediated membrane fusion. This concluded a comprehensive series of analytical and experimental efforts with identification of distinctions between HA-mediated membrane remodeling during hemifusion and fusion of bilayers (Mittal et al. 2003).

Importantly, apart from establishing Influenza HA-mediated membrane fusion as a prototypical system, the analytical and experimental advances described in this section (and shown in Fig. 2) continue to serve as reliable tools in understanding protein-mediated remodeling during membrane fusion in general, till date.

From Influenza HA-Mediated Membrane Fusion to Other Enveloped Mammalian Viruses

Table 2 lists the morphological and size variations in enveloped mammalian viruses, along with respective viral membrane proteins (VMPs, with subunits wherever applicable) responsible for receptor binding to the host and/or membrane fusion with the host. Here, an important point to note is that similar to classifications of general proteins into alpha, beta, and alpha/beta classes (Mittal and Acharya 2012; 2013), VMPs are also classified (into Classes I, II, III) based on alpha-helical and beta-sheet contents (White et al. 2008; Backovic and Jardetzky 2009; Modis 2014). Here, we do not focus on those classifications. However, we list several common themes emerging from HA-mediated membrane remodeling, which are applicable to all of viruses listed in Table 2:

1. Lipidic intermediates in protein-mediated membrane remodeling during fusion by enveloped viruses are the same (though with kinetic variations), regardless of structural variations in VMPs (Zaitseva et al. 2005).
2. All VMPs, without exception, have a "fusion peptide"—an amphipathic/hydrophobic stretch of amino acids that plays a key role in destabilizing viral and/or target membranes. Gething et al. (1986) first established the importance of this peptide by demonstrating that even a single-point mutation in HA fusion peptide renders the whole protein ineffective in membrane fusion. Length of this fusion peptide can be 10–25 residues (Bentz and Mittal 2000) and it is generally hidden/buried in the "soluble" ectodomain of VMPs before fusion. Exposure of this fusion peptide is a result of conformational changes in response to some trigger (e.g., receptor binding of VMP or lowering of pH).
3. Metastable conformations of VMPs—initial conformations of VMPs undergo irreversible conformational changes (i.e., final conformations are more "stable") resulting in membrane fusion. The paradigm of natively occurring less stable conformations in natural proteins, emerging from HA, is in contrast to expectations based on findings of Anfinsen (1972). Thus, the concept of "metastability" in the initial conformations of VMPs appropriately reconciles the post-fusion conforma-

Table 2 Enveloped mammalian viruses

Name	Family	Shape	Size (nm)	VMP(Su)	Reference(s)
Influenza Virus	*Orthomyxoviridae*	~Spherical, Pleomorphic	80–120	HA (HA1/HA2)	Elford et al. (1936), Stanley (1944), Shangguan et al. (1998), Harris et al. (2006)
FIV	*Orthomyxoviridae*	Filamentous	Len 250–30,000; Dia ~ 80	HA (HA1/HA2)	Ada and Perry (1958), Calder et al. (2010), Vijayakrishnan et al. (2013), Dadonaite et al. (2016)
NCDV	*Paramyxoviridae*	Pleomorphic	200–300	HN, F	Silverstein and Marcus (1964), Nagai et al. (1976), Ganar et al. (2014)
HPIV	*Paramyxoviridae*	Pleomorphic	150–250	F, HN	Howe et al. (1967), Hu et al. (1992), Lawrence et al. (2004)
SendaiV	*Paramyxoviridae*	Pleomorphic	150–200	HN, F(F1/F2)	Scheid and Choppin (1974)
Measles Virus	*Paramyxoviridae*	Pleomorphic, ~Spherical	120–250	H, F	Wild et al. (1991), Colf et al. (2007)
EIAV	*Retroviridae*	Pleomorphic, ~Spherical	80–120	Env	Tajima et al. (1969); Rice et al. (1990)
HIV(T1)	*Retroviridae*	~Round	~120	Env (gp160/120/gp41)	Chan et al. (1997), Harvey et al. (2007)
VSV	*Rhabdoviridae*	~Cylindrical	L ~ 190, D ~ 85	VSV-G	Kelley et al. (1972), Ge et al. (2010)
Rabies Virus	*Rhabdoviridae*	~Cylindrical	L ~ 180, D ~ 75	G	Anilionis et al. (1981), Tordo and Poch (1988)
HSV 1	*Herpesviridae*	IC, "Spikey" Envelope	~225	gB, gC, gD, gH/L	Sarimento et al. (1979), Ligas and Johnson (1988), Herold et al. (1991), Forrester et al. (1992), Grünewald et al. (2003)
EBV	*Herpesviridae*	Pleomorphic	~150–200	gH-gL-gp42 complex, gp350/220	Nemerow et al. (1987), Borza and Hutt-Fletcher (2002), Peng et al. (2010), Nanbo et al. (2018)
CMV	*Herpesviridae*	Spherical, Pleomorphic	150–200	gB, gH, gM, gL	Landolfo et al. (2003), Schauflinger et al. (2013)
SFV	*Togaviridae*	~Spherical	70	E1, E2, E3	Garoff et al. (1974), Mancini et al. (2000)
SinV	*Togaviridae*	~Spherical	~60	E1, E2	Dalrymple (1976), Choi et al. (1991)
HBV	*Hepadnaviridae*	Spherical	42	HBsAg (S, M, L)	Pasek et al. (1979), Tiollais et al. (1981)
RSV A	*Pneumoviridae*	Spherical, Filamentous	~150	G, F, SH	Mufson et al. (1985), Feldman et al. (1999)
TBE V	*Flaviviridae*	IPC*, ~Spherical	~50	E, prM (pr/M)	Chambers et al. (1990), Yu et al. (2008), Füzik et al. (2018)
DenV	*Flaviviridae*	IPC*, ~Spherical	~50	E	Chen et al. (1996), Kuhn et al. (2006)
WNV	*Flaviviridae*	IPC*, ~Spherical	~50	E, M	Mukhopadhyay et al. (2003), Kanai et al. (2006)
HCV	*Flaviviridae*	IPC*, ~Spherical	~50	E1, E2	Yu et al. (2007)
ZikaV	*Flaviviridae*	IPC*, ~Spherical	~50	E, M	Sirohi et al. (2016), Kostyuchenko et al. (2016)
OHV	*Hantaviridae*	Spherical/Oval	90–120	GN, GC	Antic et al. (1992), Xu et al. (2007)

Table 2 (continued)

Name	Family	Shape	Size (nm)	VMP(Su)	Reference(s)
VV	*Poxviridae*	Brick shaped	~360×270×250	P16, P8	Salmons et al. (1997), Johnson et al. (2006)
EbolaV	*Filoviridae*	~Cylindrical, Pleomorphic	L≥900, D~80	GP (GP1/GP2)	Volchkov (1998), Feldmann et al. (2003)
HCoV	*Coronaviridae*	~Spherical	60–140	S (S1/S2)	Bosch et al. (2003)
MERS- CoV	*Coronaviridae*	~Spherical	75–105	S(S1/S2)	Wang et al. (2013), Alsaad et al. (2018)
SARS-CoV-2	*Coronaviridae*	~Spherical	60–140	S (S1/S2)	Zhu et al. (2020), Hoffmann et al. (2020)
LFV	*Arenaviridae*	Round/Oval, Pleomorphic	100–130	GP (GP1/GP2)	Günther and Lenz (2004)
Mimivirus	*Mimiviridae*	IC-Inner membrane	400–800	GlyFP, MCP*	Xiao et al. (2005), Xiao et al. (2009), Kuznetsov et al. (2010)
ASFV	*Asfarviridae*	Icosahedral, Internal envelope	175–215	CD2v	Dixon and Chapman (2008)
TFV*	*Iridoviridae*	Hexagonal/Round	100–200	ORF001L, ORF020R	Wang et al. (2008)

ASFV African Swine Fever Virus, *DenV* Dengue Virus, *EbolaV* Ebola Virus, *EBV* Epstein–Barr Virus, *EIAV* Equine Infectious Anemia Virus, *FIV* Filamentous Influenza Virus, *GlyFP*, *MCP** Glycosylated Fibrous Proteins, Major Capsid Protein covering an inner viral membrane (host binding, viral fusion activities not yet reported), *HBV* Hepatitis B Virus, *HCoV* Human Corona Virus, *HCV* Hepatitis C Virus, *HIV*(T1) Human Immunodeficiency Virus (Type 1), *HPIV* Human Parainfluenza Virus, *HSV* 1 Herpes Simplex Virus 1, *IC* Icosahedral Capsid, *LFV* Lassa Fever Virus, *MERS* CoV-Middle East Respiratory Syndrome Corona Virus, *NCDV* NewCastle Disease Virus, *OHV* OrthoHantaVirus, *IPC** Icosahedral Protein Coat, *RSV* A Respiratory Syncytial Virus, *SARS-Cov*-2 Severe Acute Respiratory Syndrome Corona Virus2, *SendaiV* Sendai Virus, *SFV* Semliki Forest Virus, *SinV* Sindbis Virus, *TBE V* Tick-Borne Encephalitis Virus, *TFV** Tiger Frog Virus (not a mammalian virus, however is studied in a human liver cancer cell line HepG2, VMPs are putative) , *VSV* Vesicular Stomatitis Virus, *V* Virus, *VV* Vaccinia Virus, *WNV* West Nile Virus, *CMV* Cytomegalovirus, *VMP*(*Su*) Viral Membrane Protein (Subunits)

tional irreversibility with Anfinsen's thermodynamic view. This also results in "activation" and "inactivation" of VMPs—Priming for irreversible conformational changes by triggers (e.g., low pH or receptor binding) in presence of target membranes leads to membrane fusion and in absence of target membranes inactivates virus particles. Till date, only rabies virus G protein has emerged as an exception—it exhibits reversible conformational changes and can catalyze multiple rounds of fusion (Gaudin et al. 1991).

4. Occurrence of coiled coils or "*n*"-helix-bundles or hairpins in VMPs—these structural/conformational motifs are either present in pre-fusion metastable conformations or in many cases are a result of conformational changes in VMPs coupled with pre- to post-membrane fusion events (Bentz 2000b). These conformational motifs are often credited for ensuring close apposition of membranes (overcoming hydration barriers and/or generating local curvatures at fusion sites to facilitate membrane fusion).
5. Fusogenic aggregates/Fusion machines or units—multiple VMP molecules assemble together to create the required architecture of a membrane fusion site. Incomplete or different VMP assemblies lead to hemifusion or no fusion.
6. Leaky fusion—protein-mediated membrane fusion can be leaky, but only for very small molecules.
7. Data obtained from population assays for membrane fusion can be directly mapped to single-fusion events or single cell–cell fusion measurements (Mittal et al. 2002a). Interestingly, for several other systems in biology, such as single-molecule experiments, this still remains a challenge.
8. Local membrane environments (e.g., cholesterol enrichment or specific membrane domains) around VMPs are important in modulating their membrane fusion activities (Hess et al. 2005, 2007; Biswas et al. 2008; Yang et al. 2015; Lee et al. 2021).

In addition to (and preceding the above), VMPs play crucial role(s) in close apposition of viral membranes with target membranes—this close apposition requires compensating for, followed by overcoming of, the hydration barriers between the outer monolayers of the two fusing bilayers. While being a general aspect of biological membrane fusion, this involves local dehydration coupled with possible creation of transient hydrophobic defects required to be "healed" by exchange (or flip-flop) of outer monolayer lipids and/or protein fragments (Tieleman and Bentz 2002; Witkowska

et al. 2021). Here, it is also important to highlight that, other than viral membrane fusion, many of the above themes are also common to ubiquitous protein-mediated membrane remodeling events in cellular and physiological systems, such as embryonic development in worms (Gattegno et al. 2007).

Having discussed common themes on viral fusion mechanisms above, it also pertinent to view fusogenic components of VMPs (e.g., HA2 in HA) as general proteins, instead of a restricted view as only fusion proteins. Recently, it emerged that naturally occurring folded/structured proteins have clear compositional constraints (Mittal et al. 2010, 2020; Mittal and Jayaram 2011a, b). It was also shown that amino acid compositions beyond those constraints are signs of intrinsic disorder in proteins, i.e., lack of specific conformations/structures corresponding to functions (Mittal et al. 2021a, b, c). Thus, considering structural classifications in fusogenic components of VMPs (White et al. 2008), it was natural to test whether VMPs obey "stoichiometry-driven protein folding" (Agutter 2011). Figure 3 shows that VMPs are highly structured (black and yellow bars compared to striped bars in Fig. 3A), regardless of whether they are predominantly alpha-helical or beta-sheets. Also, the variability in VMP compositions, in spite of primary sequences being very different, is extremely low especially when compared to intrinsically disordered proteins (Fig. 3B). These results not only re-iterate the crucial role of relative occurrence of amino acids in naturally occurring structured proteins but also show that VMPs are highly structured with even tighter compositional constraints than general-structured proteins.

Extra- and IntraCellular Vesicles are Different from Viruses

In the context of our discussions on protein-free membrane vesicles to enveloped mammalian viruses, it becomes important to inspect other naturally occurring vesicular systems in biology. Thus, considering that enveloped mammalian

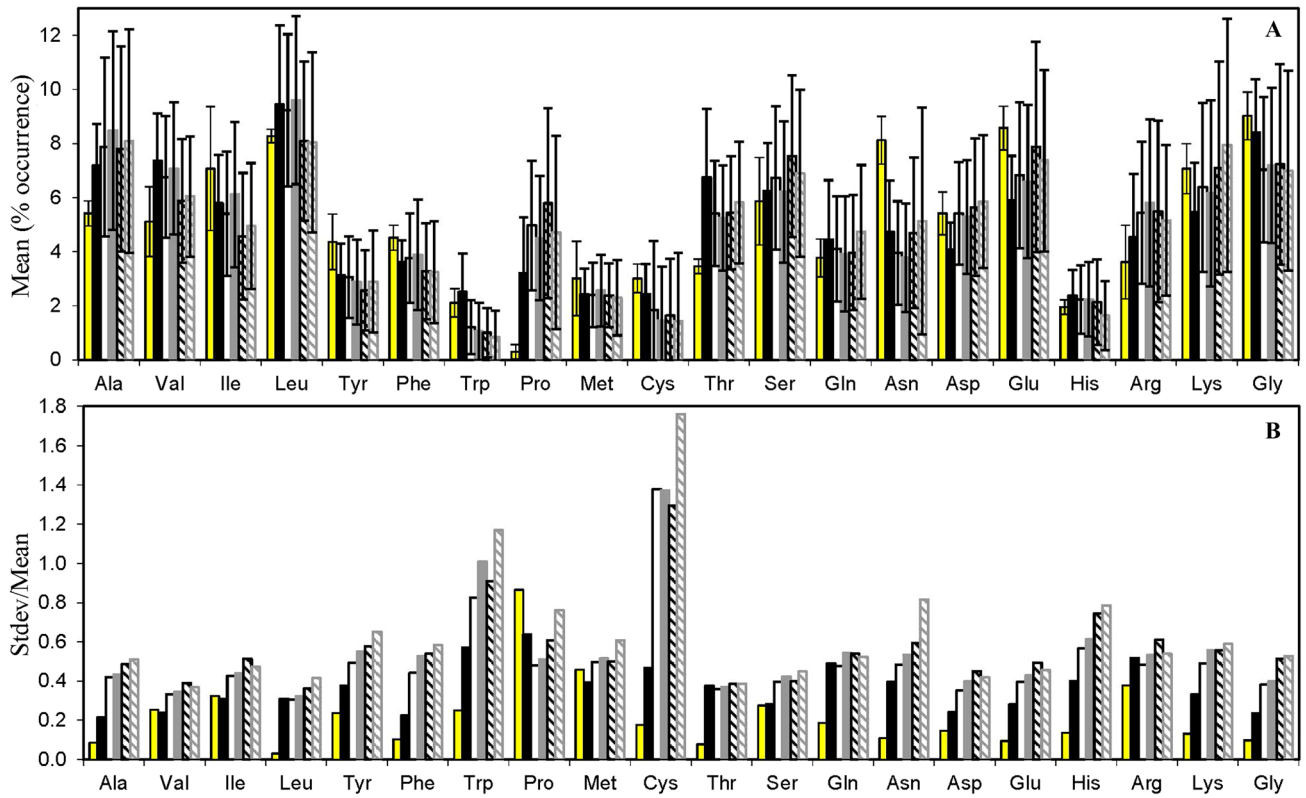


Fig. 3 Stoichiometric distributions of amino acids in viral fusion proteins (VMPs). These are compared with "structured" (open bars, $n=27{,}199$), "sequences without structure" (gray bars, $n=532{,}553$), "curated/reviewed intrinsically disordered proteins" (black-striped bars, $n=707$), and "putative intrinsically disordered proteins" (gray-striped bars, $n=94$)→for data and details, see Mittal et al. (2021c). The following sequences of fusogenic (components of) VMPs were collected from UniProtKB—HA2 (HA2-X31: P03437, HA2-Jap: P03451, HA2-PR8: P03452) and other viral fusion proteins (HIV1-gp41: P03375, HIV1-gp41: P03378, HIV2-gp41: P15831, HIV2-gp41: P20872, SFV-E1: Q8JMP5, Sin-E1:P03316, Sin-E1: P27285, TBE-E: P07720, TBE-E: P14336, TBE-E: Q01299, Den1-E: P27910, Den2-E: P29990). Yellow bars represent stoichiometric distributions of only HA2 ($n=3$) and black bars represent stoichiometric distributions of all VMPs ($n=15$)

viruses are proteo-vesicles encapsulating cargo, we take a comparative look at extracellular and intracellular vesicles.

While there are online portals dedicated to extracellular vesicles, such as "Vesiclepedia"—www.microvesicles.org (Kalra et al. 2012; Pathan et al. 2019) and "Exocarta"—www.exocarta.org (Mathivanan et al. 2012; Keerthikumar et al. 2016), Table 3 here provides a succinct, straightforward, and yet comprehensive tabulation of broad/major types of extracellular vesicles (EVs) with information only specific to the current context. It is clear that in spite of an appealing parallel between "excretory" mechanisms releasing EVs and enveloped viruses, there is one physical difference between the two—size heterogeneity within individual viruses (Table 2) is much lower than the heterogeneity observed within individual EVs. Next, a physico–chemico–biological attribute unique to enveloped viruses is uniform spatial distributions of respective VMPs on viral envelopes/surfaces independent of their shapes as well as surface density. Moreover, the variety of VMPs for individual viruses are highly limited compared to possible types of different proteins associated with EVs. Finally, while functional roles of EVs are predominantly inter-cellular communications and/or transfer of materials between cells, viruses are known to primarily hijack intracellular machinery for their own replication. In view of the above, we find that extracellular vesicles are very different from viruses. Even if their cellular sources may appear similar, mechanisms of formation and the final vesicular forms are quite distinct. We propose that it may be important to consider viruses as signatures of very primitive vesicular forms (VFs), first arising out of self-assembly of purely amphipathic constituents in aqueous environments. These VFs later associated with proteins and

Table 3 Extracellular vesicles

Name	Size (nm)	Cellular source(s)	Reference(s)
Ectosome (or microparticle/microvesicle/shedding vesicle)	50–200	Assembled and released from plasma membrane. (Neutrophils, macrophages, microglia, weaker expulsion of microparticles from other, possibly all, cell types)	Simpson et al. (2008), Cocucci and Meldolesi (2011), Van der Pol et al. (2012), Cocucci and Meldolesi (2015)
Microparticles	100–1000	Plasma membrane of most cell types	Simpson et al. (2008)
Microvesicles	20–1000	Plasma membrane of most cell types	Simpson et al. (2008), Raposo and Stoorvogel (2013)
Exosome	30–200	Exocytosis of multivesicular bodies (MVBs), rarely by plasma membrane budding	Pap et al. (2009), Van der Pol et al. (2012), Pegtel and Gould (2019)
Exosome-like vesicles	80–200	MCF-7, MDA-MB 231 cells (breast cancer)	Kruger et al. (2014)
Dexosome	~130 (50–400)	Exosomes released from dendritic cells	Näslund et al. (2013)
Argosome	Not reported	Lipoprotein particles enriched with GPI-linked proteins (exogenously derived or from plasma membranes), basolateral membranes of "Wingless-producing cells" in Drosophila	Greco et al. (2001), Vincent and Magee (2002), Panáková et al. (2005)
Epididymosome	50–250	Epididymal fluid	Sullivan (2015)
Tolerosome	~40	Intestinal epithelial cells	Karlsson et al. (2001)
Oncosome	100–400 and sometimes larger	Tumor cell membranes	Van der Pol et al. (2012), Meehan et al. (2016), Jaiswal and Sedger (2019)
Large oncosome	> 1000–> 10,000 nm	Large protrusions from/on cancer cell membranes	Di Vizio et al. (2009), Meehan et al. (2016), Jaiswal and Sedger (2019)
Prominosomes (P2, P4)	~600 (P2), 50–80 (P4)	Ventricular fluid in developing embryonic mouse brain P4, also in human colon carcinoma cells (Caco-2) and body fluids (saliva, urine, seminal fluid)	Simpson et al. (2008)
Prostasomes	50–500	Prostate epithelial cells or seminal fluid	Simpson et al. (2008)
Outer membrane vesicles	20–250	Secretory vesicles of Gram-negative bacteria	Simpson et al. (2008)

nucleic acids (or vice versa) when these chemical species became available. Subsequently, the proteo-nucleic acid-VFs became a part of chemical hit and trials eventually leading to emergence of the central dogma. Here, it is important to emphasize that the above does not support any RNA-world hypothesis. Since DNA is a chemically more stable and less reactive molecule compared to RNA (Mittal 2012), DNA viruses simply could not evolve much compared to more reactive and thus prone-to-mutation RNA viruses. In fact, the dwindling variety of DNA viruses may compel viewing them as fossilized signatures supporting the origins of life from replication of VFs, developing into proteo-DNA-VFs→proteo-DNA/RNA-VFs independent of the central dogma, subsequently transforming into living cells emerging from the central dogma.

Table 4 shows a compilation of intracellular vesicles (IVs). Almost all the points discussed in comparing EVs with enveloped viruses are applicable to comparisons of IVs with the same viruses—except that functional roles of IVs are predominantly intracellular and/or for internalization of material from outside to inside cells. Since enveloped viruses utilize intracellular trafficking pathways, transmembrane domains (TMDs) of viral VMPs show a remarkable "length" signature for intracellular organelles involved in their internalization. Thus, based on distinct membrane environments for distinct organelles inside cells (Sharpe et al. 2010; Mittal and Singh 2018), internalization pathways of enveloped viruses can be predicted based on analyzing TMDs of VMPs (Singh and Mittal 2016). Here it is important to emphasize somewhat misleading aspects of "common

Table 4 Intracellular vesicles

Name	Size (nm)	Cellular source(s)	Reference(s)
Clathrin-coated vesicle	~50, ~70, 100–120	Bud from plasma membrane and trans-Golgi network	Roth et al. (1964), Kanaseki and Kadota (1969), Pearse (1976)
COPI-coated vesicle	~75 (50–100)	Golgi-derived coated vesicle	Orci et al. (1986), Malhotra et al. (1989), Adolf et al. (2019)
COPI-coated vesicle	60–100	Bud from Endoplasmic Reticulum	Barlowe et al. (1994), Rowe et al. (1996)
Caveolar endocytic vesicle	65–90, ~100	Plasmalemmal	Peters et al. (1985), Rothberg et al. (1992)
Macropinosome	200–10,000	Plasma membrane, actin skeleton	Swanson (2008)
Phagosome	>500	Plasma membrane, actin skeleton (phagocytic cells)	Swanson (2008)
Endosome	~400 (Early) ~760 (Late)	In-budding of the plasma membrane	Ganley et al. (2004), Jovic et al. (2010), Huotari and Helenius (2011)
Lysosome	100–1200	Multi-vesicular from Endoplasm maturation in most eukaryotic cells	Lübke et al. (2009), Xu and Ren (2015), Casares et al. (2019)
Vacuole	Large, variable	Cytoplasm of plants, protists, yeasts, and some animal cells	Matile (1978), Jaquinod et al. (2007), Karunakaran and Fratti (2013), Shimada et al. (2018)
Peroxisome	100–1000	Eukaryotic cytoplasm (multiple possibilities—de novo, from ER or other peroxisomes)	Tuller et al. (1999), Terlecky and Fransen (2000), Smith and Aitchison (2013)
Apoptotic vesicle	50–5000	ER and plasma membrane in most cells	Mallat et al. (1999), Marchiani et al. (2007), Van der Pol et al. (2012), Hauser et al. (2017), Serrano-Heras et al. (2020)
Secretory vesicle	90–1000	Budding off from Golgi network	Surma et al. (2011)
Dense core vesicle/granule	~70	*trans*-Golgi network (endocrine and neuronal cells)	Kim et al. (2006), Persoon et al. (2018)
Large dense core vesicle (synaptic)	~90	*trans*-Golgi network (axon terminal-neuronal cells)	Walch-Solimena et al. (1993), Brunner et al. (2009), Gondré-Lewis et al. (2012), Kuznetsov and Kuznetsov (2017)
Small synaptic vesicle	~50	*trans*-Golgi network (axon terminal-neuronal cells)	Simpson et al. (2008)
Secretory lysosome	~700 with smaller (40–70) internal vesicles	Diverse structures (dense cores, multilaminar, unique structures) from Endoplasm maturation (in some melanocytes, cells from hematopoietic lineage, renal tubular cells)	Blott and Griffiths (2002), Griffiths (2002), Holt et al. (2006), Schröder et al. (2010), Casares et al. (2019)

features" of some IVs with viruses. Firstly, apparently both are vesicular forms with some sort of protein coats/assemblies associated with them. Secondly, already discussed previously, is the presence of common structural motifs in protein assemblies responsible for membrane fusion in both. These two need not be interpreted as similarities between IVs and enveloped viruses. The mechanisms of proteins' association with IVs are very different than those in enveloped viruses, e.g., IV protein coats have proteins that have been shown as curvature sensors; due to functional implications on cellular dynamics, protein associations with IVs are through weak interactions. In contrast, VMPs are predominantly integral membrane proteins. Additionally, the structural motifs present in viruses arise from a very few types of proteins per virus (in many cases, it is a single type of protein) compared to IVs where multiple types of proteins come together via weak interactions to form these motifs which have non-uniform spatial distributions on vesicular surfaces.

Summarizing, close inspection of the data in Tables 2, 3, and 4 indicates that neither EVs nor IVs appear to have similar proteo-vesicle-cargo formation properties with enveloped viruses. It is also important to consider that cellular vesicles are compositionally "fragile," not because of their membranes but because of reversible nature of weak interactions of proteins associated with them. In absence of data on half-lives of cellular vesicles in vivo and in vitro, a direct comparison of compositional "stability" with relatively more robust viral particles is not possible. Nevertheless, it is clear that any evolutionary linkages between viruses and cellular vesicles are premature at best and may even be biophysically unsound.

Discussion

The elegance and beauty of DNA structure offering a replication mechanism via the central dogma are undeniable. However, it is extremely difficult to visualize an accidental appearance of components of the central dogma in "dilute" solutions to initiate closely coordinated reactions for initiating life. Therefore, it is almost obvious/natural to envision appearance of replicable compartments that could encapsulate and constrain the components of central dogma to operate. Thus, origins of life must start with exploration of protein-free and nucleic acid-free replication mechanisms. These are seen in biological membranes. Recently, importance of lipid constituents of cellular "compartments" was explicitly demonstrated in origins of life and evolution of "complex" cells (Danchin 2014; Bansal and Mittal 2015). Comprehensive analyses of ~ 5000 lipid constituents of plasma membranes in the three domains of life provided direct, nucleic acids free, evidence for symbiotic origins of eukaryotic cells (Bansal and Mittal 2015). Also emerged was the reason for Archaea not being pathogens—membranes formed by sn-glycerol-1-phosphate do not fuse with membranes formed by glycerol-3-phosphate as subsequently noted by Antoine Danchin (personal communication). Clearly there is more to membrane remodeling by itself than protein-mediated membrane remodeling. In fact, it is important to consider the following:

(1) Membrane assemblies involve the same weak interactions as protein folding—these are the hydrophobic "interactions" (see legend to Fig. 1), hydrogen bonding, ionic interactions and Van der Waals forces. Of course, proteins also have two additional strong interactions (peptide bonds and disulfide linkages/bonds) that are absent in membranes—however, this absence allows morphological flexibilities.

(2) Influenza HA provided experimental evidence for "metastable" "native" protein structures to reconcile with the widely accepted thermodynamic views on protein folding beautifully illustrated experimentally by Anfinsen (1972). However, the fact that till date all experiments with biological membranes and compartments use detergents as a measure of "100%" disruptions show that membranes systems (both natural and model) are predominantly in a metastable state. Theoretically, post-disruption removal of detergent is not possible due to formation of newer equilibrium structures that would include detergent molecules. In this context, the following observations from literature are quite informative—

   a. Bentz et al. (1983b) state *It is important to mention that the equilibrium product for PS vesicles in a concentration of* Ca^{2+} *sufficient to induce fusion is a massive anhydrous structure called a cochleate ……. whose length is of the order of μm. Clearly, all of the vesicle contents will be leaked to the medium when the cochleates are eventually formed. However, the aggregation and fusion of two PS vesicles are nowhere near this equilibrium state, which is why the PS vesicle system (and other lipid mixtures such as PS/PC (phosphatidylcholine) …… can be used to study the fusion of two bilayers.*
   b. Lentz et al. (1987) state …. *kinetic analysis of our data demonstrates, first, that small vesicle preparations should be used within a few hours of size fractionation. Second, the substantial increase in fusion rate with temperature (see Figure 6) indicates a large activation energy for the rate-limiting step of the process. From our data, we estimate this to be 30–40 kcal/mol. The large magnitude of this activation energy suggests that close juxtaposition of vesicle bilayers …… may be the rate-determining*

step for spontaneous fusion of SUV. Third ….. from a practical point of view, this means that when storage is necessary, SUV should be stored just above their phase transition. Finally, we note that only the smallest, most highly curved vesicles fused to form intermediate-sized vesicles. The intermediate-sized vesicles appear to be stable above their phase transition, although our data do not rule out the possibility that these, too, fuse, but at a much slower rate than do the highly curved species.

(3) Cells and biological systems in general do not operate at optimum temperatures (or pH) for all protein constituents. There are thermodynamic windows for operation of proteins in living systems, which are not optimized toward any single protein or function (Ghosh and Dill 2010). The same is applicable to biological membranes (in terms of phase transitions and properties of individual constituents).

(4) Consideration of only thermodynamic windows for operations in living systems is also highly misleading—kinetic windows for operation of proteins, whether from the perspectives of chemical operations of the central dogma or from the perspectives of diffusion, are equally important (Dill et al. 2011). The same is applicable for "functional" membrane assemblies that, while being thermodynamically unstable (in time scales of cellular operations), have kinetic windows for stabilization by "third-party" components.

The above highlights enough reasons to consider kinetic explorations of membrane assemblies and their constituents in biological membrane remodeling, rather than relying primarily on thermodynamic considerations. Thus, we end with Fig. 4 that couples possible kinetic transitions in membranes with thermodynamics. Based on existing biological membranes, it is clear that assemblies of amphipaths are "stabilized" by proteins (and other non-lipidic components) to form dynamic, yet (meta)stable, structures away from equilibrium configurations in aqueous systems. In fact, metastability of these structures is essential for operational dynamics of living systems—a very recent example of this comes from enzymatic hydrolysis of ectodomains of membrane proteins on avian erythrocytes revealing a novel non-montonic osmotic behavior of biological membranes that stayed undiscovered in spite of nearly 100 years of literature (Singh et al. 2019). To conclude, we propose that repeated de novo formation of metastable membrane structures (i.e., mimicking replication), with "third-party" constituents (due to their availability) were not only crucial for origins of life but also continue to offer morphological replication and/or functional mechanisms in modern life forms, independent of the central dogma. In fact, very recent experimental work exploring membrane assemblies and structures lends strong support to the above proposal (Steinkühler et al. 2020, 2021).

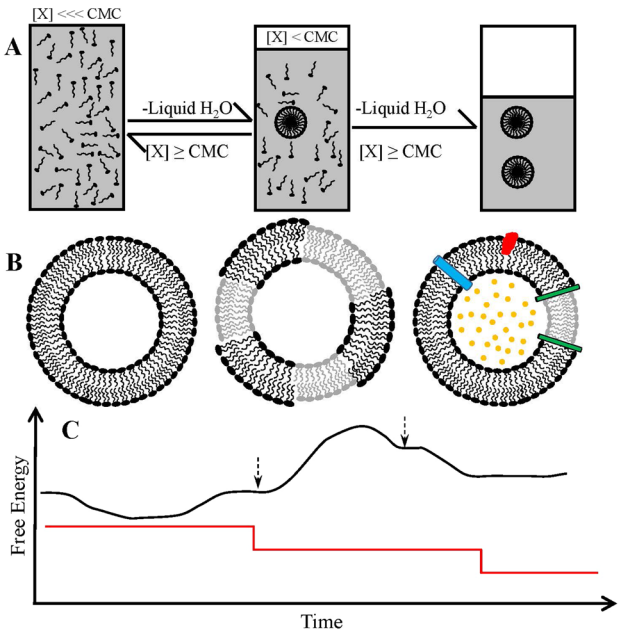


Fig. 4 Theory on origins of life and biological replication independent of central dogma. **A** De novo appearance of a micelle and "replication" of the micelle based on law of mass action—a proposal on origins of life and evolution of biological systems from a world of biological membranes (see Mittal et al. 2020 for details). **B** Unilamellar compartments with bilayer formations from similar lipids, binary mixtures, and a heterogenous systems with lipids "third-party" components. **C** Free energy diagrams for hypothetical systems shown in **A** (red) and **B** (black). The arrows represent kinetic windows during thermodynamic transitions toward stabilization of membrane formations above the possible lowest energy states. The free energy curves also represent relative free energies of respective panels in **A** and **B**. The figure is drawn only for illustrative purposes and is not to any scale

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Declarations

Conflict of interest The authors declare no competing interests.

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