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Fusogenic lipid nanocarriers: Nature-inspired design for advanced drug delivery systems

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ABSTRACT

Lipid nanocarriers are a useful tool for intracellular delivery of drugs that are otherwise unable to enter their target cells. Most of these nanocarriers are designed for an endocytotic uptake, although this route of intracellular drug delivery has a number of shortcomings. A promising alternative is fusogenic lipid nanocarriers, since by this uptake mechanism lysosomal degradation problems being associated with endocytosis can be excluded and drug release into the target cell can be synchronized with the fusion process. Various mechanisms being responsible for biological fusion events, including fertilization, exocytosis, viral infection and plasma membrane repair can be copied and transferred to nanocarriers enabling them to fuse with target cells. In particular, curvature, fluidity and surface charge of nanocarriers are key parameters for fusogenic properties. Furthermore, lipid nanocarriers can be decorated with fusogenic (poly)peptides such as viral fusion peptides or SNARE-derived lipopeptides. Within this review we provide an overview about the underlying mechanisms being responsible for cell membrane fusion processes, we demonstrate how this knowledge can be utilized for the design of fusogenic lipid nanocarriers and we summarize most promising applications of fusogenic nanocarriers for treatment of different diseases.

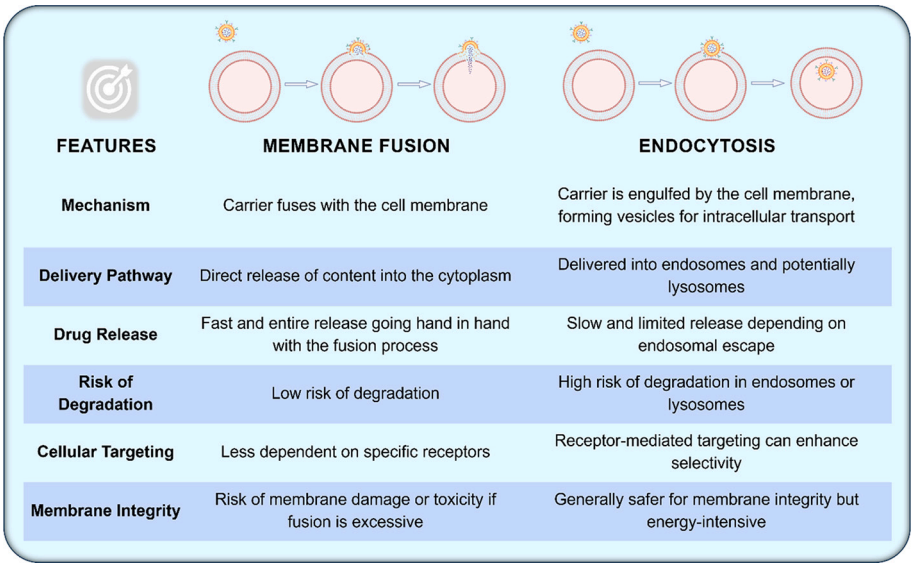
1. Introduction

Lipid nanocarriers including lipid-based nanoparticles (LNPs), solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), self-emulsifying drug delivery systems (SEDDS), liposomes and micelles are a powerful tool for mucosal and parenteral drug delivery. They can improve the solubility of lipophilic drugs, protect encapsulated drugs towards degradation, enable controlled drug release and prolong drug circulation time. Furthermore, a size $\leq$200 nm enables them to permeate the mucus gel layer covering mucosal tissues [1] and to reach underlying epithelial cells. Because of their nanoscale size they can also distribute in the tissue at the injection site getting into contact with a vast number of target cells. In addition, they can even overcome cellular membranes opening the door for intracellular drug delivery. Without lipid nanocarriers, for instance, the intracellular delivery of mRNA vaccines such as COVID-19 vaccines would not have been feasible, underlining the great therapeutic value of this type of formulations. The likely most important mechanism enabling lipid nanocarriers to overcome the cellular membrane barrier is endocytosis. This internalization mechanism, however, usually suffers from a very low delivery efficacy due to lysosomal degradation. Although various strategies have been developed to enable endosomal escape and to subsequently avoid lysosomal degradation, efficacy of drug delivery via endocytosis is often too low remaining consistently below 10 % to achieve a therapeutic effect [2]. Besides an uptake via endocytosis, however, lipid nanocarriers can fuse with cellular membranes releasing their payload into the target cell. Although the design of fusogenic lipid nanocarriers offers several advantages such as the exclusion of lysosomal degradation problems and a drug release going hand in hand with the fusion process as summarized in **Fact Box 1**, formulators are just rarely making use of this 'contact-facilitated uptake mechanism designed by nature'. It is therefore the aim of this review to provide a detailed overview about this mechanism and the various strategies how it can be employed for the design of fusogenic lipid nanocarriers. Furthermore, we summarize the most promising applications of fusogenic lipid nanocarriers for treatment of different diseases and take an outlook on future trends.

Fact Box 1 Membrane fusion and endocytosis as cellular entry pathways, (Created with BioRender.com).

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FEATURES	MEMBRANE FUSION	ENDOCYTOSIS
Mechanism	Carrier fuses with the cell membrane	Carrier is engulfed by the cell membrane, forming vesicles for intracellular transport
Delivery Pathway	Direct release of content into the cytoplasm	Delivered into endosomes and potentially lysosomes
Drug Release	Fast and entire release going hand in hand with the fusion process	Slow and limited release depending on endosomal escape
Risk of Degradation	Low risk of degradation	High risk of degradation in endosomes or lysosomes
Cellular Targeting	Less dependent on specific receptors	Receptor-mediated targeting can enhance selectivity
Membrane Integrity	Risk of membrane damage or toxicity if fusion is excessive	Generally safer for membrane integrity but energy-intensive

2. Cell membrane fusion – A fundamental physiological process

Cell membrane fusion is an essential process in various biological events, including fertilization, exocytosis, viral infection and plasma membrane repair [3–6]. The field of membrane fusion research has greatly evolved from the early proposals of the stalk and hemifusion intermediate stages to the more recent continuum models describing membrane fusion as a progressive remodeling of the system [7]. These theoretical models focus on the physicochemical properties of the lipid bilayers rather than the conformational changes of fusion (poly)peptides, providing a general framework to understand the energy barriers and driving forces in membrane fusion. Yet, many molecular level mechanisms being involved in fusion process in the complex biological environment, especially the internalization process, are uncertain [8]. As illustrated in Fig. 1 membrane fusion is a multistep process that requires the removal of the aqueous environment associated with the polar headgroups and the contact between the membranes, known as stalk. The following local bilayer disruption induces a lipid mixing in the outer leaflets, with the formation of a hemifusion diaphragm defined as hexagonal phase (H_{II}), which is a non-bilayer lipid configuration characterized by lipids arranged in long, cylindrical structures, forming a hexagonal lattice when viewed in cross-section, and finally opening of a fusion pore [9,10].

This process is highly regulated but can be influenced by key parameters including curvature, fluidity, charge and surface (poly)peptides as illustrated in **Fact Box 2** [12,13]. Furthermore, particular features of the aqueous environment such as pH and ion concentration can impact parameters like fluidity, charge and hydration of the membrane, indirectly influencing the fusion process [14–16]. Understanding these parameters is essential in unraveling the intricate mechanisms of membrane fusion. Most of these key parameters, being essential for fusion process, can be adapted for design of fusogenic nanocarriers.

Fact Box 2 Overview about key factors for membrane fusion.

KEY FACTORS	OVERVIEW
Curvature	• Curvature influences the energy required for the fusion process. It can be controlled by lipid composition of nanocarriers • The fusion process is predisposed by **negative** curvature and inhibited by **positive** curvature
Fluidity	• Increased **fluidity** enhances the fusion process • In contrast, **rigidity** increases stability avoiding aggregation and fusion phenomena
Surface charge	• **Positively charged** lipid nanocarriers are attracted by the **negatively charged** cell membrane • This mechanism can trigger the fusion of lipid nanocarriers with target cells
Surface (poly)peptides	• Specific peptide patterns can trigger the **protein-mediated proximity** between lipid nanocarriers and cell membrane • Targeted fusion with certain cell types can be enabled by (poly)peptide triggers

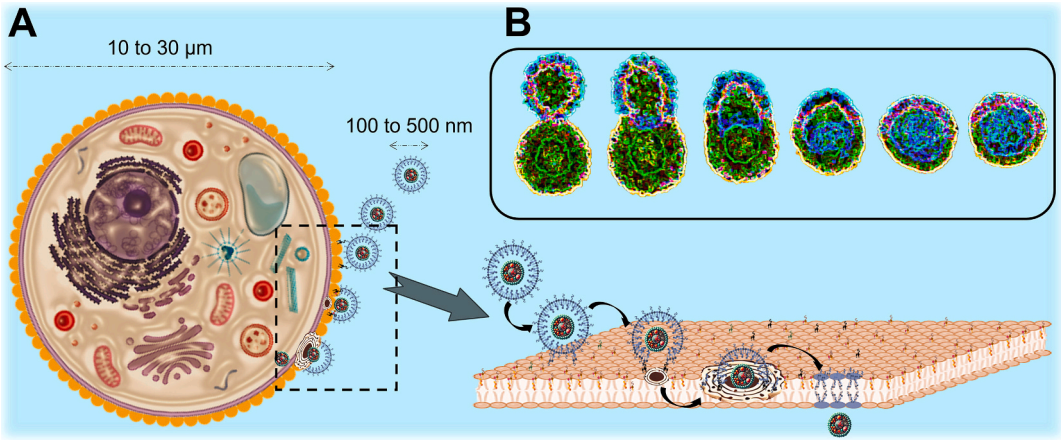


Fig. 1. A. Illustration of membrane fusion process, **B.** The structural changes over the course of fusion event; outer leaflets contact, stalk, hemifusion pore appears, full fusion, Adapted from [11], Copyright (2012), The royal society of chemistry.

2.1. Curvature

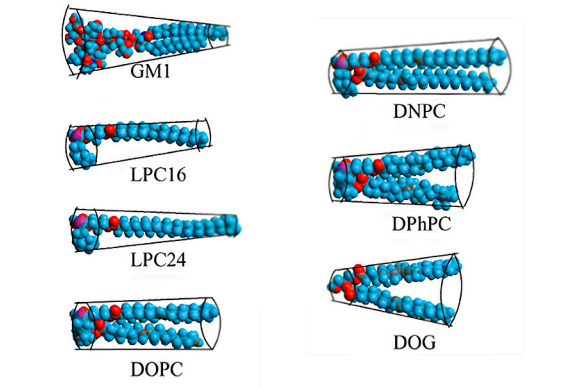
The lipid composition of nanocarriers is one of these key parameters being essential for fusion, as lipids with specific geometries can either promote or inhibit the formation of intermediate structures such as the stalk and hemifusion diaphragm. In this regard, membrane curvature is essential for cell membrane fusion, as it reflects the intrinsic shape of lipids [10,17]. Lipids of different shape and "intrinsic curvature" can induce varying degrees of membrane curvature, which in turn affects the formation of fusion intermediates [18].

The complex interplay among the lipids involved in fusion processes is difficult to fully describe, nonetheless it is possible to identify lipids with a higher headgroup-to-hydrophobic acyl chain ratio and intrinsic positive curvature, such as lysolipids, as potential inhibitors of the fusion process since they hinder stalk formation due to their cone-like shape [19–21]. Lipids with a small headgroup and large hydrophobic tails, such as phosphatidylethanolamine (PE), have an inverted cone shape and promote negative curvature, facilitating stalk formation [22,23], whereas, lipids with a cylindrical shape, such as phosphatidylcholine, generate little curvature, which can hinder fusion [24,25].

In Fig. 2 the impact of surfactants with cone shape, cylindrical shape, and inverted cone shape on the fusogenic properties of nanocarriers measured via their lipid mixing reflecting mainly formation of the initial fusion intermediate (stalk) and contents mixing reflecting conversion of this intermediate either to a second intermediate or to a fusion pore is shown [26].

2.2. Fluidity

Fluidity of lipid nanocarriers is another major parameter involved.



* **GM1,** ganglioside (ovine brain)
* **LPC16,** 1-palmitoyl-2-hydroxy-3-sn-phosphocholine
* **LPC24,** 1-lignoceroyl-2-hydroxy-3-sn-phosphocholine
* **DOPC,** 1,2-dioleoyl-3-sn-phosphatidylcholine
* **DNPC (C24),** 1,2-dinervonoyl-3-sn-phosphatidylcholine
* **DPhPC,** 1,2-diphytanoyl-3-sn-phosphatidylcholine
* **DOG,** dioleoylglycerol

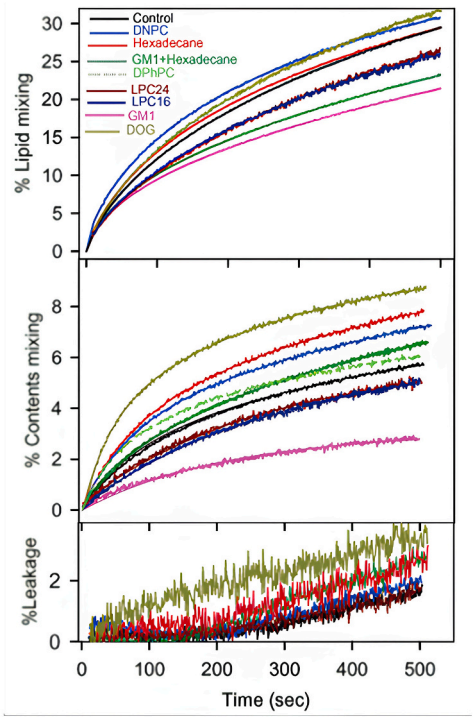


Fig. 2. Lipid mixing reflects mainly formation of the initial fusion intermediate (stalk), while content mixing reflects conversion of this intermediate either to a second intermediate or to a fusion pore. Content leakage reflects that it varies depending on lipid composition. Adapted with permission from [26]. Copyright (2004) American Chemical Society.

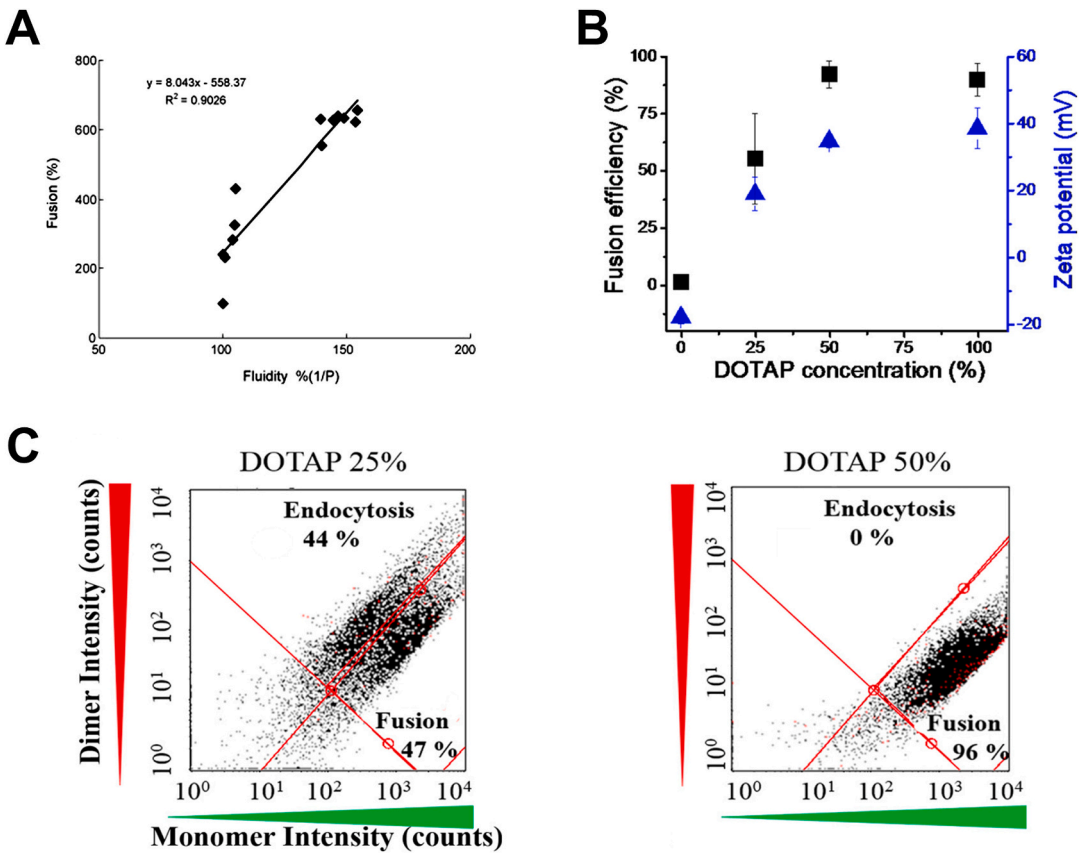


Fig. 3. **A.** Relationship between membrane fluidity and fusion activity of various ceramide-based liposomes; adapted from Tokudome et al. [33] with permission from Elsevier, **B.** Liposomal zeta potential (blue triangles) and fusion efficiency (black squares) at different cationic lipid concentrations, with bars showing standard deviations, **C.** Flow cytometry dot plots showing cellular uptake pathways and their efficiencies. Liposomes contained the BODIPY FL-DHPE tracer, with its monomer signal detected in the green and dimer signal in the red channels after incubation with CHO cells. Endocytotic uptake displayed nearly equal monomer and dimer tracer signals, while membrane fusion showed high monomer and low dimer signals. Reproduced under terms of the CC-BY license [38]. Copyright 2018, MDPI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

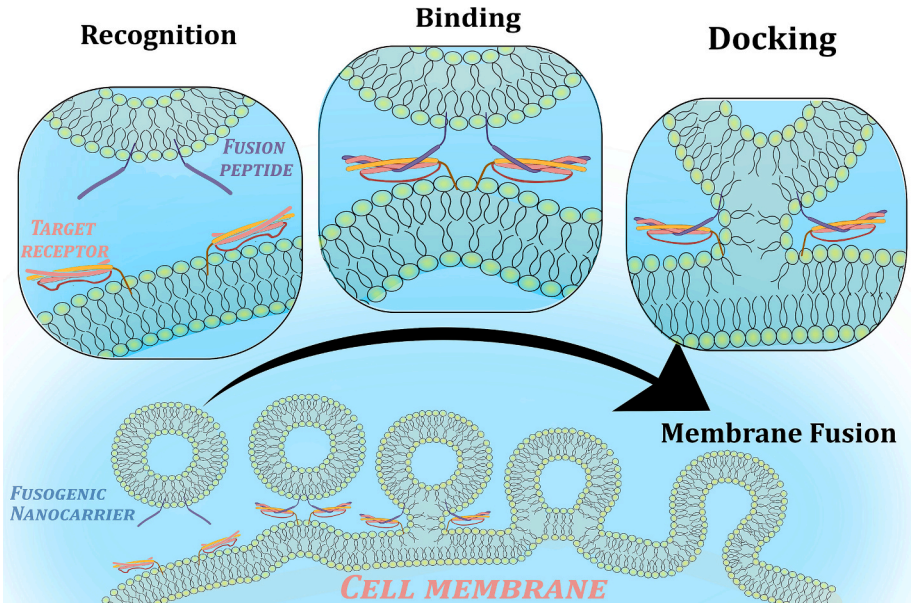


Fig. 4. Key steps for (poly)peptides mediated membrane fusion between nanocarrier and cell membrane. The mechanism exploits the interactions between specific receptors on the cell membrane and fusogenic peptide to mimic the designed-by-nature of fusion already observed for viruses and extracellular vesicles.

Lipid rafts are an ideal target as cholesterol-rich platform in cell membranes that are thought to play important roles in various cellular processes, such as signal transduction and intracellular trafficking. They are characterized by a liquid-ordered (L_o) phase, which is more tightly packed and less fluid than the surrounding cholesterol-rich liquid-disordered (L_d) phase [27]. Due to the differences in lipid packing and order, there is a height mismatch at the interfaces between coexisting L_o and L_d phases. This height mismatch results in an interfacial energy called "line tension" [28]. The presence of lipid rafts and their associated line tension can significantly impact membrane fluidity [29]. The rigid nature of the L_o domains reduces the overall fluidity of the membrane in those regions [30]. However, the boundaries between the L_o and L_d phases, where line tension is highest, can exhibit increased fluidity and flexibility and were identified as the best site for fusion, in contrast with the non-raft domain [31,32]. This apparent counterintuitive phenomenon can be explained by the fact that the bending and non-bilayer lipid intermediates required for fusion are energetically favored at the L_o-L_d phase boundaries [28]. Tokudome et al. prepared liposomes composed of C-8 ceramide, cholesterol, linolenic acid and cholesterol sulfate in different ratios resulting in low, medium and high membrane fluidity of these lipid nanocarriers. As illustrated in Fig. 3A, the membrane fluidity of these liposomes directly correlated with their fusion activity [33].

2.3. Charge

Additionally, the presence of anionic lipids like phosphatidylserine in the target cell membrane can cause electrostatic repulsions that prevent the close contact between membranes, thus impeding the fusion process [34,35]. As cellular membranes display always an anionic surface charge, anionic lipid nanocarriers are repulsed from their surface [36,37]. This parameter can be investigated by evaluating the impact of zeta potential of lipid nanocarriers on their fusogenic properties. In fact, the more positive the surface charge of lipid nanocarriers, the higher is the fusion efficacy and internalization. Kolašinac et al., for instance, could show that there is a direct correlation between the zeta potential of liposomes and their fusogenic properties until a plateau phase is reached at a zeta potential $\geq +40$ mV. As illustrated in Fig. 3B-C, the more DOTAP was added to these lipid nanocarriers, the more positive was their surface charge and the higher was their efficacy to fuse [38].

2.4. Surface (poly)peptides

(Poly)peptide-mediated membrane fusion has been developed to mimic the mechanisms that viruses and certain intracellular vesicles use to fuse with cellular membranes. Furthermore, fusion (poly)peptides (fusogens) play a crucial role in enhancing membrane fusion between cells by bringing cell membranes closer together, disrupting lipid bilayers, facilitating hemifusion, and guiding the formation and expansion of the fusion pore [4]. Generally, the (poly)peptide-mediated membrane fusion process can be divided into several stages as illustrated in Fig. 4: recognition and binding, docking, hemifusion, and full fusion. Fusion (poly)peptides first recognize and bind to specific receptors on the target cell membrane, ensuring that the lipid nanocarrier targets the correct cells [39]. During docking, the nanocarrier is brought closer to the target membrane through interactions between fusion (poly)peptides and cell receptors [10]. Next, the outer leaflets of the nanocarrier and target membrane merge to create a hemifusion diaphragm, and finally, the inner leaflets mix to form a fusion pore, allowing the drug to be released into the cytoplasm [40,41].

This mechanism can be facilitated in particular by coiled-coil (poly) peptides [42,43]. They act as engines, generating intrinsic forces that lead to membrane fusion by establishing hydrophobic connections and undergoing conformational changes upon encountering specific cellular receptors or environmental triggers like pH changes or calcium ions [6,44]. The change in the (poly)peptide quaternary structure due to the increase in helix content observed by circular dichroism (CD) spectroscopy indicates the coiled-coil formation [45]. E/K coiled-coil peptides provided valuable mechanistic insights into the fusion process between peptides and lipid membranes. This is due to the dual affinity of Kn peptide (KIAALKE)n for both the En peptide (EIAALEK)n and fluid phospholipid membranes. When the peptide K binds to the membrane, it induces positive curvature, destabilizing the membrane and facilitating fusion [46]. Researchers also indicate that the length of the (poly)peptide used to modify the carrier surface significantly affects the performance of the fusogen. These (poly)peptides help lipid nanocarriers to merge with target cell membranes by overcoming the plasma membrane and energy barriers [47,48]. Fig. 5 shows the effect of (poly)peptide length and concentration on the fusogenic properties of lipid nanocarriers measured through lipid mixing at constant total lipid concentration.

Various fusion (poly)peptides, including viral fusion (poly)peptides and soluble N-ethylmaleimide-sensitive factor attachment receptor

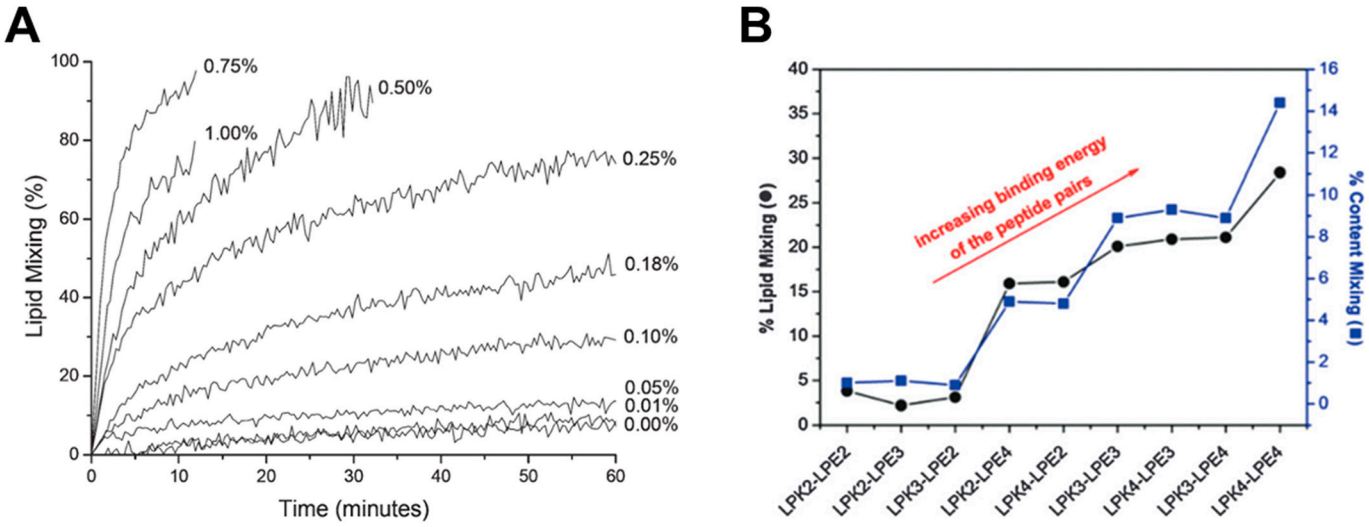


Fig. 5. A. Total lipid mixing was recorded for increasing lipopeptides (LPE and LPK) at a fixed concentration of total lipids in liposomes, **B.** Correlation of lipid and content mixing to the coiled-coil lipidated peptide pairs with increasing stability. Reprinted with permission from [49,50]. Copyright (2013) The Royal Society of Chemistry.

(SNARE) proteins were shown to play specific roles in this mechanism [51–53]. Viral membrane fusion has been adapted for intracellular drug delivery, as viruses have evolved mechanisms to deliver their genomes to host cells [54,55]. For instance, Sendai virus envelopes have been used to create fusogenic virosomes for drug delivery [56,57]. Similarly, the presence of fusion (poly)peptides such as SNARE proteins, or their derivatives [58–60] in fusogenic formulations, is crucial in facilitating various stages of membrane fusion between nanocarriers and cell membranes. These (poly)peptides enhance targeting specificity, destabilize lipid bilayers to promote fusion and regulate the timing and location of fusion [19,61].

Synthetic peptides mimicking influenza virus hemagglutinin, such as INF5 and INF7, destabilize membranes in a pH-dependent manner and have been incorporated into liposomal drug formulations to enable endosomal escape and release into the cytosol of tumor cells [62]. GALA is one of the engineered peptides designed to mimic the function of viral fusion (poly)peptides sequences. It is a pH-sensitive, amphipathic peptide characterized by repetitive Glu-Ala-Leu-Ala motifs [63]. GALA is highly effective in facilitating the release of calcein from cholesterol-free liposomes. One design involves pH-sensitive liposomes with fusogenic peptides, such as GALA, that undergo conformational changes at low pH [64]. These peptides remain monomeric and water-soluble at neutral or high pH but become hydrophobic and form a transmembrane helix structure at acidic pH, integrating into the membrane and facilitating liposome-cell fusion. This mechanism is useful in low pH environments like tumors or inflamed tissues, enhancing drug release into cells [65–67]. Another peptide, EGLA, is formed by replacing an alanine residue in GALA with glycine. Although EGLA is less active than GALA, it still exhibits significant leakage activity in cholesterol-containing membranes [64,68,69].

3. Technologies to study fusion processes

In order to gain deeper insight into the underlying mechanisms of cell membrane fusion, researchers have employed a range of experimental methods and techniques. These experiments aim to provide evidence for different stages of the fusion process between cell membranes and formulations, primarily liposomes, ranging from initial membrane contact to complete content mixing. In vitro membrane fusion model systems have been extensively studied to address formulation challenges and explore potential solutions. Given the intricate nature of membrane fusion, which is challenging to observe and manipulate directly in biological systems, numerous in vitro models have been developed in recent years. These models provide researchers with a simplified environment to control and investigate the fusion process in greater detail [8,70].

3.1. Fluorescence techniques

Fluorescence techniques have been established as the primary method of demonstrating fusion, due to their versatility in utilizing a diverse range of suitable fluorescent dyes that can be incorporated into both the cell membrane and the lipid nanocarriers. This approach enables the observation of the inclusion of the fluorescent dyes in the cell membrane or the cytosolic uptake of the loaded dyes within the cell, preventing any occurrences of leakage. Moreover, these techniques allow for the quantitative assessment of the fusogenic properties of lipid nanocarriers, thereby facilitating improvements in their efficacy.

In particular, FRET-based assays employ a pair of fluorescent probes, a donor and an acceptor, wherein the emission spectrum of the donor coincides with the excitation spectrum of the acceptor. When the donor and acceptor are in close proximity, excitation of the donor results in

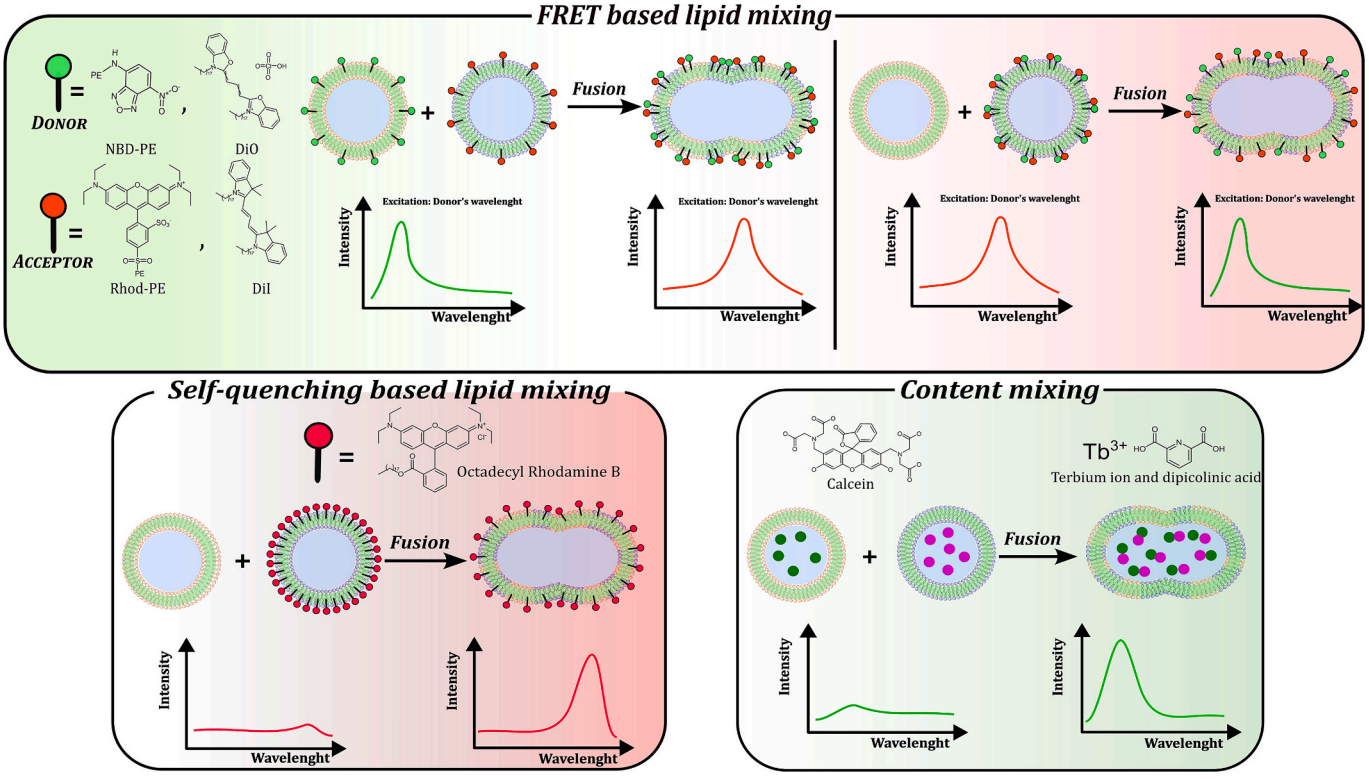


Fig. 6. In vitro techniques for the detection of fusion phenomena using fluorescent dyes are able leverage spatial interaction with other fluorescent molecules. In the example illustrated, liposomes serve as model nanocarriers. FRET-based techniques exploit the close spatial arrangement of donor and acceptor fluorophores: upon fusion, the reduced distance between the fluorophores results in enhanced emission from the acceptor, indicating successful interaction. Conversely, when the FRET pair is embedded within the nanocarrier formulation, fusion with a target membrane results in dilution of the fluorophores, leading to an increased intermolecular distance and a consequent reduction in FRET efficiency and acceptor fluorescence. Self quenching techniques and Content mixing techniques are based on the same approach.

energy transfer to the acceptor, which in turn leads to acceptor fluorescence [71]. This approach permits the provision of evidence for fusion when the acceptor, present in the cell membrane, displays an elevated intensity signal resulting from its closer proximity to the donor, loaded into the lipid nanocarrier. By contrast, in instances where fusion has taken place with lipid nanocarriers that are loaded with both the acceptor and the donor, the distance between the FRET pair is increased, while the signal emitted by the acceptor is reduced (see Fig. 6). It is therefore essential that the dyes are specifically localized within the cell membranes or liposomes that are used to mimic cell membranes. This ensures that any signal changes that are observed can be attributed to events that occur as part of the process of cell fusion, rather than to other interactions that take place within the cell [70,72].

The lipid mixing assay can be based on this approach or on the use of a FRET pair. The formulations are loaded with a fluorescent lipid at a certain concentration. Upon fusion with the cell membrane, the fluorescent lipids dilute into the larger membrane structure and, depending on the experimental setup, differences will be observed in terms of fluorescence. In fact, FRET experiment can be focused on the loss of signal of the acceptor when both acceptor and donator are loaded in the formulation, due to the dilution and increased distance between the dyes [73]. An alternative approach involves incorporating the donor and acceptor into the cell membrane and lipid carrier, respectively. Upon fusion, this enables the probes to come into close proximity, resulting in a FRET signal [74]. This signal change confirms lipid mixing between the lipid nanocarrier and cell membrane [70,75,76].

Some of the most commonly utilized FRET combinations in these scenarios include nitrobendiazole (NBD) and rhodamine derivative dyes, which have undergone chemical modification to target specific parts of the membrane [77,78]. Another possibility is the adoption of cyanine compounds, likewise DiI/DiO pair (1,1′-dioctadecyl-3,3,3′3’-tetramethylindocarbocyanine perchlorate/ 3,3′ -dioctadecyloxacarbocyanine, perchlorate), known for their spectral overlap and ability to act as a FRET pair while also localizing to the cell membrane [79]. Generally, different combinations are possible depending on the spectral overlapping [80].

Another possible approach is to exploit the phenomenon of fluorescence quenching observed at high dye concentrations. This approach was pioneered by Hoekstra et al. [81] using octadecyl rhodamine B as a fluorescent dye included in their system. Alternatives involving the use of fluorescein derivatives have also been discovered, which are capable of almost completely self-quenching their fluorescence at concentrations exceeding 100 mM.

Similar to lipid mixing assays, the content mixing assays use encapsulated fluorescent probes to monitor the fusion process. Liposomes are loaded with a water-soluble fluorescent dye at a self-quenching concentration Tb^{3+} /dipicolinic acid (DPA), calcein [82–84]. Fusion with the cell membrane allows the dye to escape into the cytosol, leading to dilution and an increase in fluorescence signal. This observation confirms the mixing of aqueous contents between the liposome and the cell, indicative of complete fusion [85].

Confocal Laser Scanning Microscopy (CLSM) allows for the visualization of fluorescently labeled lipid nanocarriers and cell membranes. By labeling these components with different fluorescent probes, researchers can track nanocarrier's interaction with the cell membrane over time. Colocalization of fluorescent signals suggests membrane fusion. This technique is particularly useful in visualizing the distribution of lipid nanocarriers within cells, distinguishing between membrane fusion and endocytic uptake [86–88]. The fluorescent dyes used are the ones described above for the lipid mixing assay. Their use alone or as FRET pair allows qualitative and quantitative observation of the membrane fusion process.

Fluorescence Recovery After Photobleaching (FRAP) assesses the lateral diffusion of fluorescently labeled molecules within membranes. In the context of membrane fusion, a region of the cell membrane containing fluorescently labeled lipids is photobleached, eliminating the fluorescence signal. If fusion occurs between the cell membrane and lipid nanocarriers containing the same fluorescent lipid, the bleached region will show fluorescence recovery over time as the labeled lipids diffuse from the lipid nanocarriers into the cell membrane [89].

Flow cytometry analyzes the optical properties of single cells in a fluid stream. In membrane fusion studies, cells are treated with fluorescently labeled formulations and then analyzed by flow cytometry. Cells that have undergone fusion will exhibit a shift in their fluorescence signal due to the acquisition of fluorescent lipids from the lipid nanocarriers [90,91]. This method allows for the quantification of the percentage of cells that have undergone fusion and it can be combined with FRET to better discriminate the fusion processes from other internalization pathways [92].

3.2. Other techniques

Differential Scanning Calorimetry (DSC) measures the heat flow associated with phase transitions in lipid bilayers. By analyzing the thermograms of liposomes before and after incubation with cells, researchers can infer changes in the lipid organization caused by membrane fusion [36]. For example, the disappearance or shift of a phase transition peak suggests the integration of liposomal lipids into the cell membrane [93–95].

Furthermore, the use of specific inhibitors for different internalization pathways allows to discriminate between fusion and endocytosis (Table 1). In particular, the fusion process can be inhibited by cholesterol depleting compounds such as methyl-β-cyclodextrins. Fusion inhibiting peptides take advantage of structural moieties that, in contrast to fusion inducing (poly)peptides, are able to selectively interact with fusion (poly)peptides and inhibit the process [96,97]. Incubation of cells at 4 °C inhibits energy-dependent cellular uptake mechanisms such as endocytosis [98,99], whereas fusion process can still take place. Cellular uptake studies of lipid nanocarriers at 4 °C versus 37 °C enables, in particular in combination with specific inhibitors, to discriminate between endocytotic and fusogenic uptake mechanisms.

A comparative study with non-fusogenic cationic liposomes was conducted to demonstrate the cellular uptake efficiency of K4-dimers as a fusogenic coiled-coil peptide. The study was carried out both at 4 °C and 37 °C and in the presence of endocytosis inhibitors (NaN_3, MβCD, wortmannin). The experiments with cationic liposomes revealed that their uptake is energy-dependent, primarily driven by macropinocytosis and dependent on lipid raft integrity. In contrast, fusogen peptide-decorated liposomes facilitate uptake through membrane fusion, which occurs independently of energy consumption but still requires lipid integrity [46].

Another technique to study fusion processes is transmission electron microscopy (TEM). Wang et al. were able to elucidate the different steps of fusion through different specimens utilized TEM as illustrated in Fig. 7.

These experimental approaches, often used in combination, provide a comprehensive picture of the membrane fusion process. Each technique offers unique insights into different aspects of fusion, contributing

Table 1
Specific inhibitors for different routes.

Inhibitor	Route	Ref.
Methyl-β-Cyclodextrin	Endocytosis; membrane-fusion	[59,100]
Chlorpromazine	Clathrin-mediated endocytosis	[101]
Amiloride	Macropinocytosis-mediated endocytosis	[102]
Nystatin	Caveolae-mediated endocytosis	[103]
NaN_3	Energy-dependent endocytosis inhibitor	[104]
Wortmannin	Endocytosis, inhibits phosphoinositide 3-kinase	[105,106]
Z-Phe-Phe-Phe-OH	Membrane-fusion	[96]

to a deeper understanding of the underlying mechanisms. It is important to note that no single methodology provides a complete understanding of membrane fusion. A combination of techniques is often required to fully characterize the process and to obtain comprehensive insights into the underlying mechanisms.

pros and cons of fusogenic lipid nanocarriers are summarized in **Fact Box 3**.

Fact Box 3 Advantages and challenges of fusogenic lipid nanocarriers.

ADVANTAGES	
Reduced toxicity and improved biocompatibility	Mimics biological membranes, reducing adverse reactions
Bypasses degradation pathways	Enhances cellular uptake
Versatility in therapeutic cargo	Can carry various therapeutic agents like small molecules, genes and proteins
Protection from degradation	The lipid nanocarriers shield encapsulated agents from enzymatic or environmental damage
Controlled drug release	Allows synchronized cellular uptake and release
CHALLENGES	
Complex mechanisms	Uncertain behavior in biological environments
Off-target effects	Potential interactions with unintended cell types or components, complicating targeted delivery
Premature release risk	Vulnerable to losing encapsulated drugs before reaching the target
Strict testing requirements	Requires extensive evaluation to meet safety and efficacy standards
Tracking difficulties	Challenges in visualization and monitoring within in vivo settings

4. Design of fusogenic lipid nanocarriers

Efficient delivery into cells without damaging cellular membranes is crucial for the therapeutic effect of many drugs. In fact, many drugs cannot permeate the lipid bilayer of cell membranes because of their hydrophilic character, charge and size. The development of fusogenic formulations starts from the idea to mimic this designed by nature mechanism with the objective to obtain formulations capable of efficiently fusing with cell membranes. The unique mechanism of fusion, which bypasses the typical endocytic pathway, offers several benefits:

Firstly, the direct delivery of the cargo to the cytoplasm provides a higher concentration of the drug at its intracellular target site and improves the bioavailability and thus the therapeutic efficacy of the encapsulated drug.

Secondly, the fusogenic feature allows for the delivery of a wider range of payloads, including larger molecules such as proteins and nucleic acids, which are otherwise difficult to deliver efficiently due to their poor membrane permeability [108,109].

Thirdly, the direct fusion with the cell membrane can enhance the kinetics of cellular uptake compared to the slow and multistep endocytic pathways, thus potentially improving the therapeutic onset [74,110].

Finally, the fusogenic nature of the delivery system can enable targeted delivery to specific cell types or tissues by incorporating cell-specific ligands or peptides that promote membrane fusion [111]. The

Additionally, lipid nanocarriers are generally biocompatible, which helps in reducing immune response. The versatility of lipid nanocarriers allows them to be engineered to carry a wide variety of therapeutic molecules, including hydrophobic and hydrophilic small molecules, DNA, RNA, and proteins [71,78]. They are particularly beneficial for improving the solubility of lipophilic drugs, thereby increasing their bioavailability. The lipid core of these nanocarriers also protects encapsulated drugs from degradation by shielding them from enzymatic or environmental factors. Moreover, lipid nanocarriers can be designed to enable controlled drug release, allowing for sustained therapeutic effects and reducing the frequency of dosing. Table 2 highlights how specific lipids, peptides, and responsive materials facilitate contact-triggered or stimulus-induced fusion with cellular membranes.

Toxic risks are another challenge that must be carefully addressed. The lipid composition, charge, and size of nanocarriers need to be precisely controlled to avoid cytotoxicity, which could harm healthy cells. Furthermore, ensuring the compatibility of the therapeutic payload with the lipid matrix without compromising the fusion ability adds to the formulation's complexity. There is also a risk of unintended interactions between lipid nanocarriers and non-targeted cells or components, which can affect the fusion process and therapeutic outcomes.

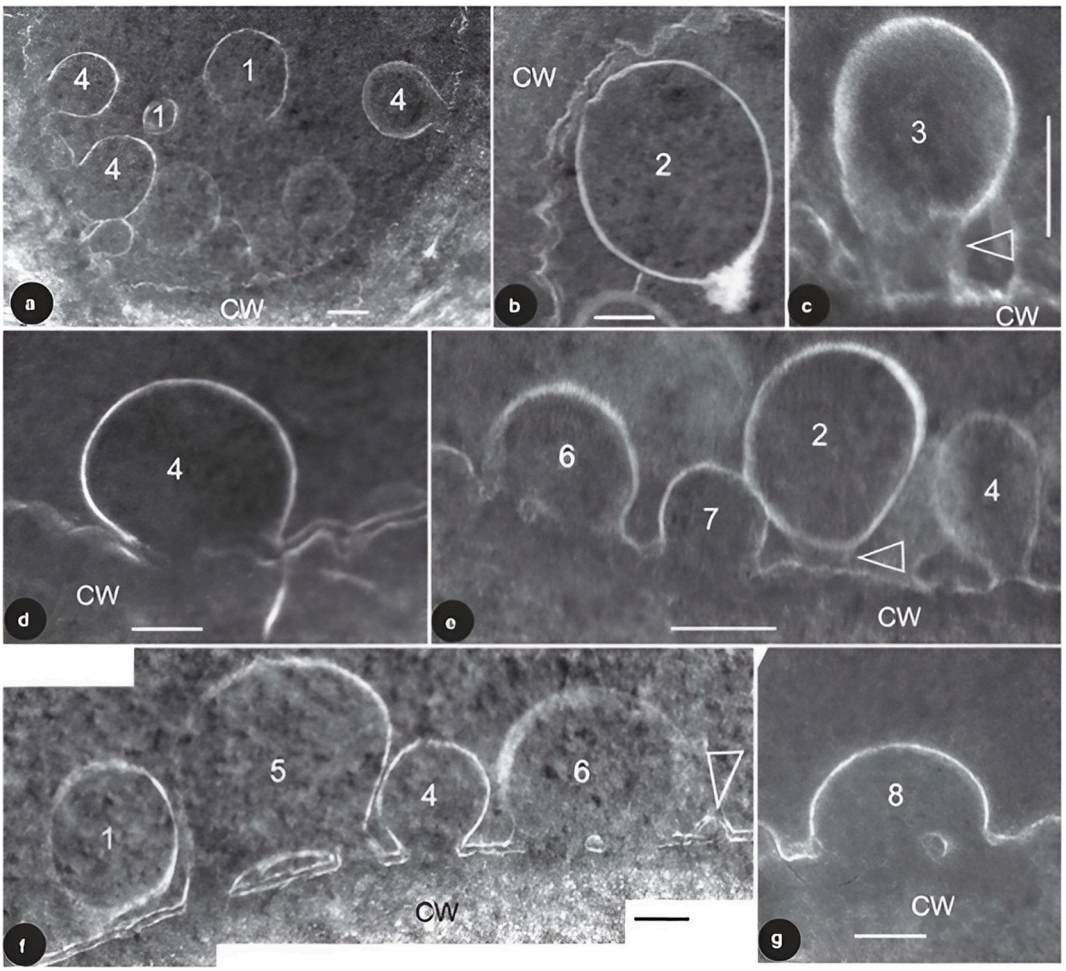


Fig. 7. Fusion stages are numbered. CW = cell wall. Scale: 100 nm. (a) About nine vesicles in various fusion stages within the same cell; S1 vesicles are separate from the cell membrane (CM), while S4 vesicles are fused. (b) A vesicle near the CM with a spherical shape, showing slight deformation near the CM. (c) A vesicle connected to the CM by a cylindrical link (arrow). (d) A fused vesicle, where its membrane attaches to the inner leaflet of the CM. (e) Four vesicles in different fusion stages: in S2, weakly tethered; S4, connected by a narrow neck; S6, omega-shaped with a central plug; S7, with a wider fusion opening and no plug. (f) Four vesicles with the CM's double-layered structure. S1 is separate, S5 has a central plug, S6 a remnant plug, and S4 lacks an outer CM leaflet. (g) A vesicle almost fully fused with the CM. Reproduced under terms of the CC-BY license from [107]. Copyright (2011) Taylor & Francis.

4.1. Excipients used for the design of fusogenic nanocarriers

Lipid nanocarriers that exploit curvature for fusion frequently employ the use of lipids that possess intrinsic curvature or are capable of promoting curvature in the membrane (INSERT Fig. **8**.

Fig. 8). Liposomes comprising dioleoylphosphatidylethanolamine (DOPE), a lipid with negative curvature, show considerable fusion efficiency [117]. DOPE is a neutral lipid that assists in membrane destabilization due to its small headgroup, which causes it to adopt a hexagonal conformation [118]. The conical shape of DOPE can stabilize the intermediate hemifusion stage structure formed during fusion [19]. In addition to the lipid composition, other factors, such as particle size can

Table 2
Design strategies of fusogenic lipid nanocarriers to promote membrane fusion.

Component / Strategy	Role in Fusion	Examples	Key Considerations	Ref.
Cationic lipids	Enhance electrostatic interaction with negatively charged cell membranes. Often combined with helper lipids to optimize fusion and reduce side effects.	DOTAP, DOTMA (constitutively positively charged)	Toxicity, charge density	[38,85]
Helper lipids	Stabilize lipid bilayer or modulate fluidity	DOPE (fusogenic), cholesterol (modulates membrane fluidity and curvature).	Bilayer rigidity vs. flexibility	[29,75]
Peptide, protein ligands, mimicking viral fusion or fusogenic peptides	Mimic viral fusion mechanisms or facilitate membrane recognition through biological motifs	Viral fusion peptides (influenza HA2), TAT, GP41, coiled-coil peptides	Fusion specificity, immunogenicity	[112,113]
pH-sensitive or stimuli-responsive design	Enhanced membrane interaction, destabilization, and ultimately fusion with the endosomal membrane under physiological triggers such as pH or temperature	Ionizable lipids, pH-sensitive linkers/polymers	Controlled release, intracellular targeting	[114,115]
Lipid phase behavior tuning	Promote non-lamellar phases that support fusion	Inverted hexagonal (H_{II}) phase-forming lipids	DOPE: It is a workhorse fusogenic lipid. Its propensity to form HII phases is key.	[113,116]

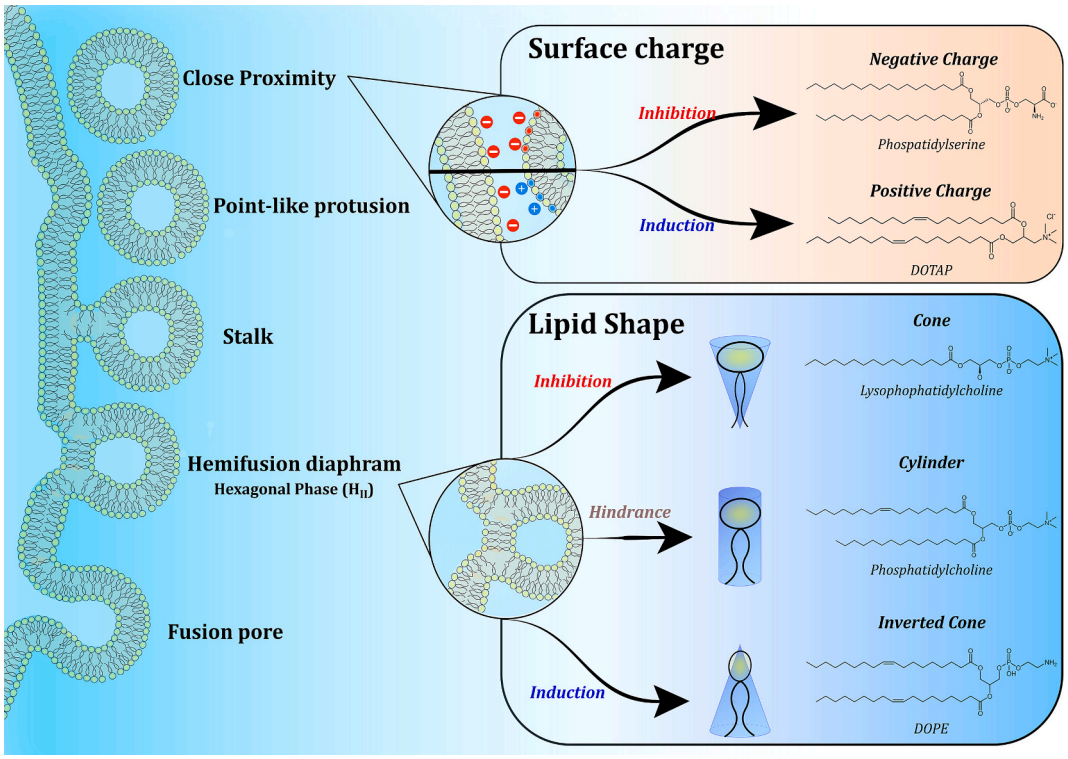


Fig. 8. Schematic representation of the main strategies for the development of (poly)peptides-free drug delivery systems and relative target step. Two major physicochemical parameters modulate fusion efficiency: surface charge and lipid shape. Positively charged lipids (e.g., DOTAP) promote membrane fusion throughout the attraction of nanocarriers and cell membrane, whereas negatively charged lipids (e.g., phosphatidylserine) inhibit it because of the repulsion between negatively charged membranes. Similarly, lipid molecular shape affects fusion dynamics: inverted cone-shaped lipids (e.g., DOPE) favor the induction of non-lamellar intermediates and facilitate fusion, while cone-shaped lipids (e.g., lysophosphatidylcholine) inhibit fusion, cylindrical lipids (e.g., phosphatidylcholine) hinder progression of fusion because of their geometrical shape.

also influence fusion efficiency. For example, it has been observed that smaller particles with higher curvature fuse more easily with target membranes [119].

In contrast, lipids with a positive intrinsic curvature, such as lysophosphatidylcholine (LPC), have been demonstrated to inhibit fusion processes. This is due to the conical shape of these lipids, which hinders the formation of the hemifusion intermediate [76]. The capacity of membranes to undergo fusion is also contingent upon the lipid composition of the inner and outer leaflets. For instance, the oligomerisation of lipids exclusively within the inner leaflet of the bilayer is sufficient to impede fusion, as it diminishes the mobility of the lipid headgroup within the bilayer, thereby influencing the ease of lateral lipid diffusion, which is indispensable for fusion [11]. Membrane fusion is a process that necessitates membrane flexibility and typically occurs above the phase transition temperature of the bilayers.

The incorporation of lipids that promote membrane fluidity has been demonstrated to enhance membrane fusion. This increased fluidity permits the reorganization and fusion of membranes with greater ease. Cholesterol, unsaturated lipids and lipids with short acyl chains exploit an analogous mechanism in promoting the membrane fusion lowering the phase transition temperature and increasing fluidity [77,120]. To illustrate, in the context of influenza, elevated cholesterol levels diminish the energy required to form the hemifusion stage by modifying the curvature of the host membrane. In the case of HIV, cholesterol facilitates fusion between cholesterol-poor and cholesterol-rich disordered domains of target membranes. The incorporation of cholesterol into liposomes can reduce their phase transition temperature, resulting in a more fluid membrane at lower temperatures. This is attributed to cholesterol's interference with the dense packing of fatty acid tails in the lipid bilayer [19,81,93].

An alternative strategy adopted in fusogenic formulations exploits the properties of cationic lipids to enhance contact phase, facilitating the fusion between the lipid nanocarriers and cell membrane (INSERT FIG. 8. Fig. 8). In this regard, 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) represents the gold standard. DOTAP is a cationic lipid that induces membrane fusion through electrostatic interactions with the negatively charged cell membrane [119].

One major concern with fusogenic formulations, especially those containing cationic lipids like DOTAP, is their potential for cytotoxicity. Cationic lipids can disrupt cell membranes, affecting membrane charge homeostasis and potentially leading to cell death [38,76,121]. However, the inclusion of helper lipids like DOPE in the formulation can mitigate the toxicity associated with cationic lipids [122,123]. The extent of cytotoxicity can also depend on the specific cell type being targeted. For instance, macrophage cell lines, with their inherent phagocytic function, may exhibit higher endosomal accumulation and potential toxicity compared to other cell lines [76].

In addition to DOPE and DOTAP, other lipids are mentioned in the context of fusogenic lipid nanocarriers or factors impacting membrane fusion, such as aromatic lipids that can significantly enhance the fusogenicity of lipid nanocarriers when combined with DOPE and DOTAP.

Table 3
Drug delivery through membrane fusion using engineered (poly)peptide formulations.

(Poly)peptide	Nanocarrier/Cargo	Target	Effect/Aim	Ref.
Coiled-coil forming peptides "E_3" and "K_3"	Liposomes/Doxorubicin	Zebrafish embryos and HeLa cell line	Targeted membrane fusion for rapid and efficient drug delivery	[126]
Coiled-coil peptides N-acetylated peptides	Liposomes/Proflavine hydrochloride	LN229 cell line	Coiled-coil peptides as pH sensitive triggers enhancing liposomal drug delivery	[132]
Coiled-coil forming peptides "E_4" and "K_4"	Liposomes/Bis-(thioureido)decalin	Giant unilamellar vesicles (GUVs) and Fischer rat thyroid (FRT) cell line	Coiled-coil-driven membrane fusion: efficient system for delivering anion transporters to target cell membranes	[133]
Coiled-coil forming peptides "E_4" and "K_4"	Cuboidal mesoporous silica nanoparticles/Cytochrome-C (cytC)	HeLa cell line	Membrane fusion for efficient cytosolic delivery of cytC, promoting apoptosis in cells	[134]
Octa-Arginine peptide (R8)	Macrophage-mediated liposomes/ Paclitaxel (PTX) and resveratrol (Res)	4 T1 cell line and Female BALB/C mice	Dual-guide strategy using membrane fusion for both targeting and therapeutic effects	[135]

The aromatic component characterized by an extended conjugated π electron system, such as the one present in many fluorescent dyes, can be attached to either the headgroup or the chain region of the lipid molecule [38,85].

4.2. Fusogenic (poly)peptides used for the design of fusogenic lipid nanocarriers

The incorporation of engineered fusion (poly)peptides improves the lipid nanocarriers' ability to destabilize lipid bilayers, allowing for efficient lipid exchange and ultimately mixing with cell membranes. Lipid nanocarriers are decorated with simplified versions of SNARE proteins, designed as lipidated oligopeptides (coiled-coil peptides) connected to a transmembrane domain, which anchors the (poly)peptides to the lipid membrane [124]. Some research on membrane fusion properties of nanocarriers having been decorated with fusogenic (poly)peptides are presented in Table 3.

The synthetic lipopeptides are usually composed of a hydrophobic tail, a linker, and (poly)peptides serving as fusogenic recognition motifs. Typically, (poly)peptide-mediated fusogenic lipid nanocarriers are created by combining lipids and lipidated (poly)peptides in an organic solvent, such as chloroform [125]. Regarding the lipid moiety used in these lipopeptide structures, DOPE and especially cholesterol were reported to provide fusion efficiency. Thanks to cholesterol, lipidated (poly)peptides can be easily anchored into any lipophilic core of nanocarriers [126]. The hydrophobic tail provides the efficient immobilization of the (poly)peptide at the surface of the nanocarriers [127,128]. Furthermore, (poly)peptide length has also a direct impact on fusion efficiency. The coiled-coil binding strength and (poly)peptide-membrane interactions increase with increasing (poly)peptide length [50].

The activity of these (poly)peptides depends on factors such as (poly)peptide sequence, lipid composition of the lipid nanocarriers, and the pH of the medium. For instance, GALA is effective in cholesterol-free liposomes but less so in those containing cholesterol. Recent research has shown that coiled-coil-based systems and SNARE-derived lipopeptides can induce membrane fusion and enhance drug uptake, particularly for targeting cancer cells [49,59,129].

In a study demonstrating the ability of lipidated coiled-coil-forming peptides to induce membrane fusion, the effect of different lipid components on fusion efficacy was evaluated by lipid and content mixing experiments (Fig. 9A-B). Cholesterol and DOPE were demonstrated to mediate membrane fusion with high efficacy [125].

Polyethylene glycol (PEG) is often employed as a flexible and variable length linker to regulate the distance between bilayers, keeping the (poly)peptide near the membrane and maintaining colloidal stability. Additionally, the photolabile PEGylation of lipid nanocarriers membranes enables precise control over the timing and location of fusion events, utilizing PEG as a steric shielding agent between fusogenic (poly)peptides and lipid nanocarriers [43,45,130]. Another advantage of using PEG is to prevent the penetration of fusogen into normal cells by utilizing the PEG hydration layer on the surface of lipid nanocarriers [131].

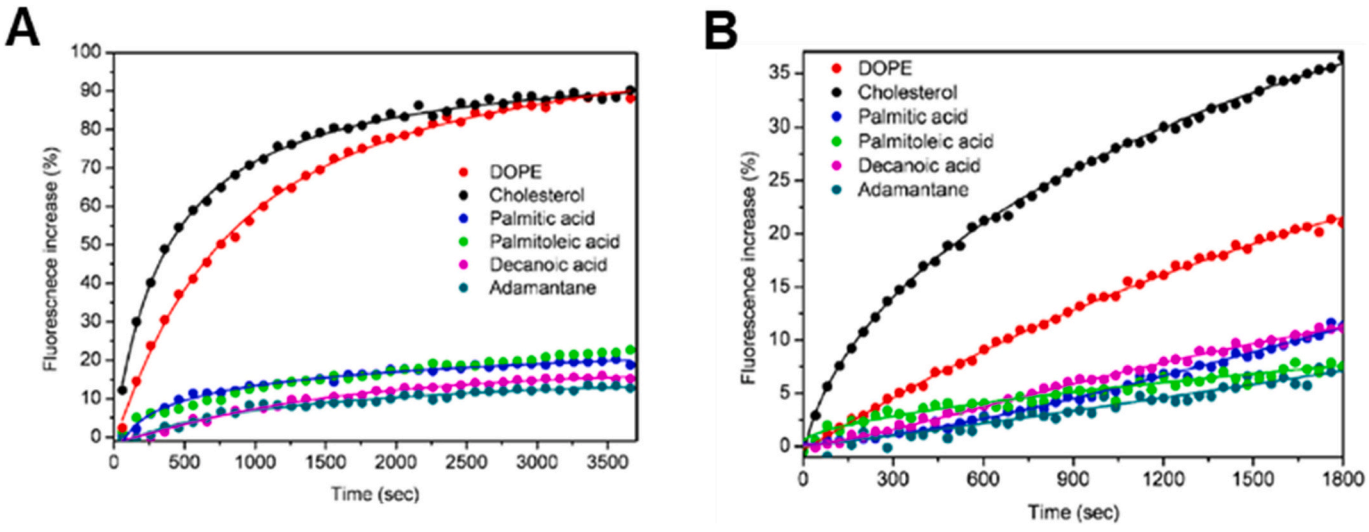


Fig. 9. Comparison of the fusion efficiency measured via increase in fluorescence of liposomes having been decorated with fusogenic peptides that were anchored via spacers of increasing chain length, **A.** Lipid mixing between peptide E and peptide K decorated liposomes, **B.** Content mixing between peptide E and peptide K decorated liposomes [125], Reprinted with permission from [125]. Copyright (2013) American Chemical Society.

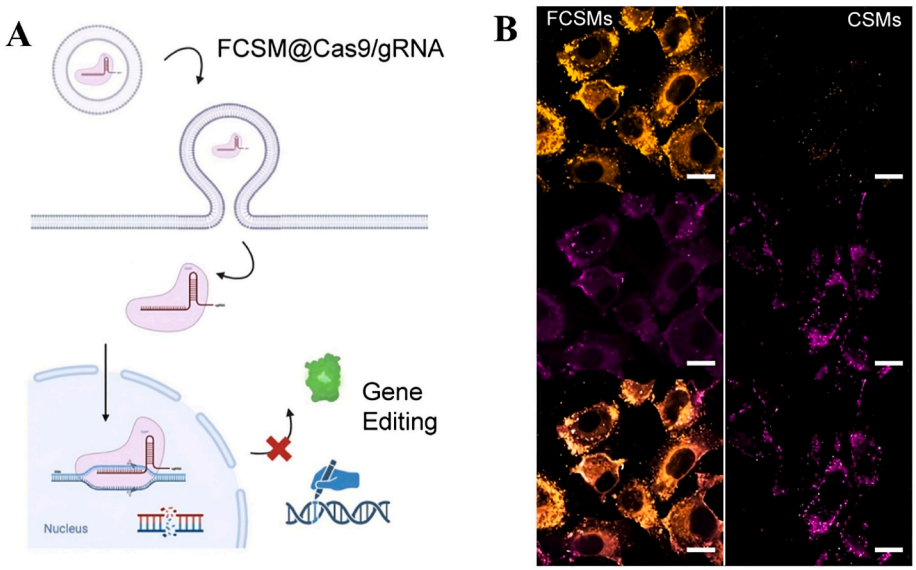


Fig. 10. A. Schematic representation of CRISPR/Cas9 gene editing delivered by a fusogenic liposome (FCSMs). **B.** Confocal images of A549 cells incubated with fusogenic (left panel) and non-fusogenic (CSMs) liposome marked with dextran-SNARF (DS) for 10 min. In each panel, the columns display fluorescence signals as follows: DS (orange, top row), DOPE-647 N-labeled (F)CSM (violet, middle row), and the overlay of both fluorescence channels (bottom row). Scale bars: 20 μm. Reproduced under terms of the CC-BY license [141]. *Copyright (2023), Elsevier. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)*

5. Application of fusogenic lipid-based nanocarriers for treatment of different diseases

5.1. Anticancer drugs formulations

The application of fusogenic formulation in cancer therapy could represent a transformative approach in enhancing the therapeutic efficacy and safety profiles of various pharmaceuticals. Indeed, the fusion uptake pathway enables the direct introduction of chemotherapeutic drugs into the cytosol, circumventing the biological barriers that impede their access to their target [117]. In Fig. 10, Soprano et al. combined the cell membrane-derived nanocarriers and fusogenic excipients for the delivery of different cargos through the cytosolic route. This strategy enables direct fusion with the plasma membrane, irrespective of the nature of the cargo. By integrating the biomimetic characteristics of the nanocarriers, such as natural homotypic targeting, with their fusogenic capabilities, intracellular delivery was successfully achieved for a wide range of payloads, including small molecules, large non-cell-permeant macromolecules, and solid nanoparticles. The main advantage was obtained by circumvents the endosomal route of internalization and the action of efflux pumps, which impede the attainment of a therapeutic concentration due to degradation and elimination [136,137].

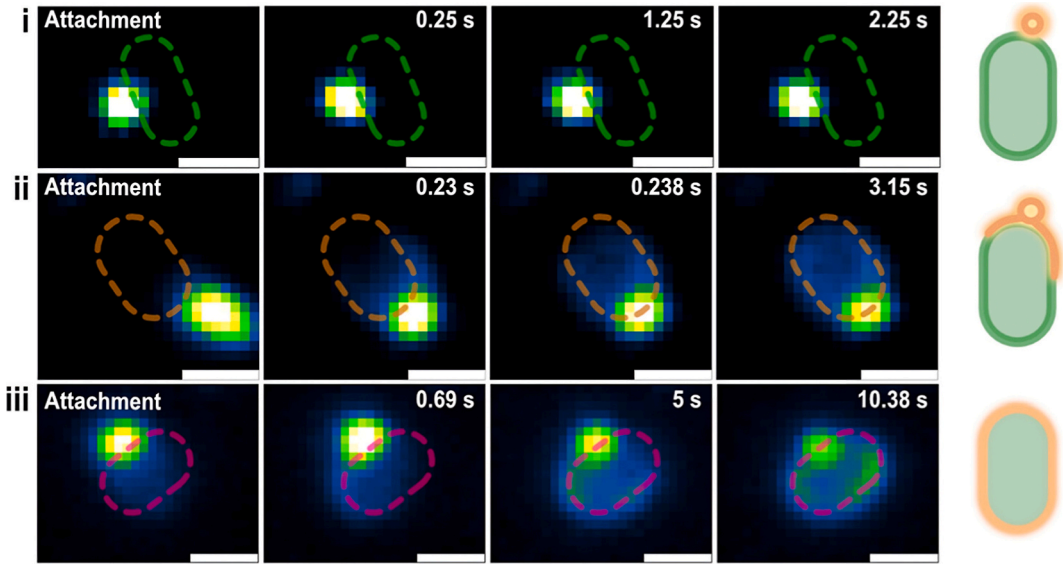


Fig. 11. Interactions of fluorescent fusogenic liposomes with poly L-lysine-immobilized BL21 cells visualized using laminated optical sheet microscopy (HILO): time-lapse imaging revealed three distinct interaction patterns. These include **(i)** attachment of liposomes to the bacterial perimeter without noticeable changes in fluorescence intensity, **(ii)** attachment accompanied by partial diffusion of Rhodamine-PE into the outer membrane (OM), leading to a fluorescence intensity reduction of less than 50 % from the peak value, and **(iii)** complete liposome integration, characterized by uniform Rhodamine-PE diffusion throughout the OM and minimal residual fluorescence at the attachment site. A schematic representation of the three interaction stages is shown on the right. Reproduced under terms of the CC-BY license [144] *Copyright (2023), MDPI.*

Bleomycin, an antitumor drug requiring cytosolic delivery for optimal therapeutic action, has been incorporated into fusogenic liposomes modified with octarginine (R8-DOPE-BLM) with up to 50-fold improvement in terms of cellular uptake when compared to unmodified liposomes, demonstrating significantly stronger cell death and DNA damage in vitro compared to conventional formulations. These modified liposomes exhibited also a notable antitumor effect in BALB/c mice bearing 4 T1 tumors, suggesting their potential for enhanced cancer therapy [138]. Additionally, paclitaxel, a widely used chemotherapeutic agent, has been investigated in combination with doxorubicin within fusogenic and long-circulating liposomes, showing enhanced antitumor activity and modulated toxicity in murine models of breast cancer [139]. The variety of drugs loaded in fusogenic delivery systems underscore the importance of intracellular delivery for these drugs and how fusogenic liposomes can significantly enhance drug uptake and efficacy [140]. The advancement of fusogenic liposomal formulations represents a significant leap forward in drug delivery technology, with the potential to greatly improve therapeutic outcomes in cancer treatment by enhancing intracellular delivery, efficacy, and safety of various drugs.

5.2. *Antimicrobial drug formulations*

Fusogenic lipid nanocarriers have emerged as a promising approach also for antimicrobial therapies for their ability to merge with bacterial cell membranes, facilitating the direct delivery of their payload into the bacterial cytoplasm. The fusion with bacterial cell membranes and biofilms can be achieved by the use of the fusogenic lipids already adopted for eukaryotic cells. In fact, the cationic and auxiliary lipids of the nanocarriers promote bacterial adsorption, enhancing the bactericidal action and increasing membrane disruption and permeability [142,143]. Although many studies have already been published in this regard, the specific interactions between individual nanocarriers and bacterial targets remained insufficiently understood. In their study, Scheeder et al. employed high-resolution microscopic techniques to elucidate the stepwise fusion process of optimally formulated fusogenic nanocarriers in both Gram-positive and Gram-negative bacteria confirming the presence of three key phases of membrane fusion as illustrated in Fig. 11: initial membrane contact, hemifusion formation, and, consequently, complete fusion. Notably, fusogenic liposomes interact differently with Gram-negative and Gram-positive bacteria. In Gram-negative bacteria, the liposomes merge with the outer membrane, facilitating membrane fusion. In contrast, in Gram-positive bacteria, their interaction is primarily characterized by attachment or lipid internalization [142,144].

A notable study investigated the therapeutic efficacy of a cationic bilayer nanoemulsion for the topical delivery of fusidic acid to eradicate MRSA 33591 infections in burn wounds on a murine model. The cationic bilayer nanoemulsion loaded with fusidic acid was incorporated into a 2

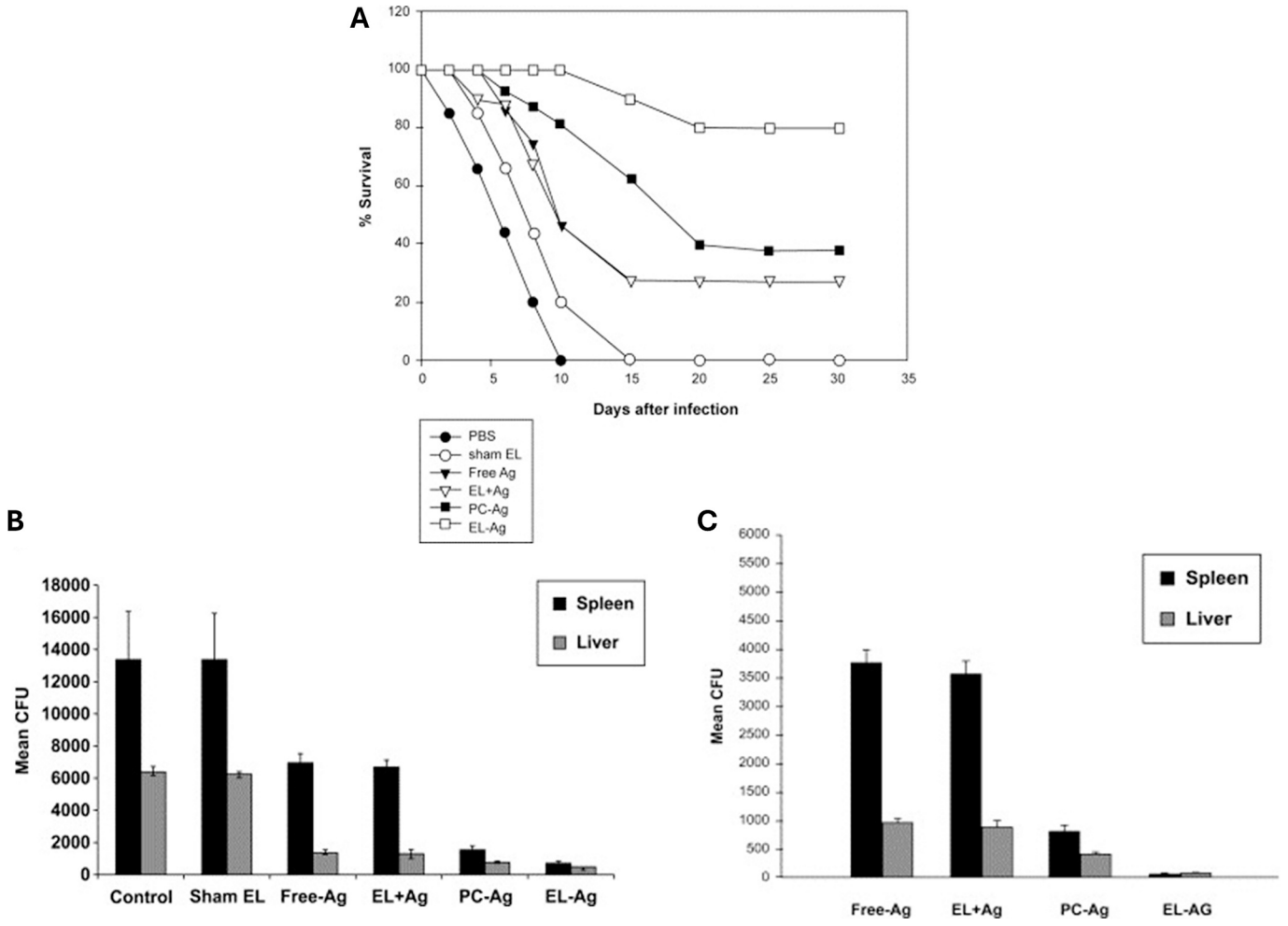


Fig. 12. **(a)** The prophylactic potential of fusogenic liposome (Sham-EL or EL) was assessed through survival rates in animals challenged with S. typhimurium infection after vaccination. Sham-EL empty fusogenic liposome, EL-Ag fusogenic liposome loaded with antigen, PC-Ag non fusogenic liposome with antigen. **(b)** The effect of immunization on infection establishment was evaluated by measuring bacterial loads in key organs on day 7 post-challenge. **(c)** Fusogenic nanocarriers efficacy was further evaluated by assessing bacterial burdens in organs on day 15 post-infection. Animals immunized with PBS or empty liposomes did not survive to this point. Reprinted with permission from [149] Copyright (2006) Elsevier.

% *w/v* Carbopol 940 hydrogel characterized through rheological and texture analysis and applied for wound healing. Antibacterial activity assays showed that the bilayer nanoemulsion loaded with fusidic acid and the cationic bilayer nanoemulsion loaded with fusidic acid had lower minimum inhibitory concentration values compared to the drug in ethanolic solution and cream form [145]. Alternatively, Kim et al. used porous silicon nanoparticles with a homing peptide as their fusogenic formulation to target macrophages and induced stronger response against *Staphylococcus aureus* in pneumonia patients. Moreover, the use of fusogenic formulations is not limited to the field of small molecules; they can also be employed in the delivery of biological macromolecules [146]. Mamusa et al. demonstrated that POPC/DOPE liposomes decorated with 12-bis-THA (1,1′-(dodecane-1,12-diyl)-bis-(9-amino-1,2,3,4-tetrahydroacridinium) are able to efficiently deliver transcription factor decoys (TFDs) into the cytoplasm of *Escherichia coli*, highlighting the potential in delivering oligonucleotides [147]. Although promising, fusogenic liposomal formulations show some cytotoxicity, suggesting the need for further optimization to modulate their therapeutic index.

5.3. Vaccines

Among therapeutic strategies, vaccination is increasingly used in many fields to harness the patient's own immune system to fight microbial infections and tumors. Fusogenic nanocarriers used for vaccine can release their contents directly into the cytoplasm of target cells, a critical feature for inducing a robust cell-mediated immune response [148,149]. This strategy is crucial for the activation of the cytotoxic T lymphocytes (CTLs) that require antigens to be presented via the class I major histocompatibility complex pathway, which is characterized by the need of the antigens in the cytosol [150,151]. Fusogenic liposomes have been shown to induce stronger CTL responses compared to non-fusogenic liposomes or traditional adjuvants, such as complete Freund's adjuvant. For instance, studies involving cytosolic antigens from *Salmonella typhimurium* encapsulated in fusogenic liposomes demonstrated significant reduction of proliferation of the bacteria in mice and increased survival rate (Fig. 12), indicating robust CTL activation [149]. Moreover, while CTL induction is a hallmark of fusogenic liposomes, they also have the capability to trigger both humoral (antibody-mediated) and cell-mediated immune responses, as seen with HIV-1 gp120 V3 loop peptides encapsulated in pH-sensitive liposomes, which induced specific virus-neutralizing antibodies and CTL responses. The composition of fusogenic liposomes can be tailored to optimize antigen delivery and immunogenicity, with the presence of fusogenic lipids such as DOPE enhancing their membrane fusion ability and induction of IgG2a antibody responses, indicating a Th1-type immune response [151,152]. Efficient antigen encapsulation in cationic liposomes influences the immune response, skewing it towards a Th1 profile, while incorporating adjuvants like monophosphoryl lipid A further enhances immunostimulatory properties by activating antigen-presenting cells via Toll-like receptor 4 [148,151]. Taken all, fusogenic liposomes represent a significant advancement in drug delivery technology, particularly in immunotherapy, by enhancing intracellular delivery, inducing strong CTL responses, and activating both humoral and cell-mediated immunity, making them a promising platform for vaccine development and cancer immunotherapy.

5.4. Other applications

Besides applications mentioned above, fusogenic nanocarriers are also tested for various other applications. Sirolimus, for instance, has been encapsulated in fusogenic liposomes to improve its therapeutic profile, with studies demonstrating superior immunosuppressive activity compared to conventional liposomal formulations. Specifically, research has shown that fusogenic liposomes significantly enhance T cell proliferation inhibition, highlighting their potential in improving the efficacy of sirolimus in immunosuppressive therapies [153].

6. Future trends

Since different cell types exhibit pronounced differences in the lipid composition of their cellular membranes and consequently in membrane fluidity, fusogenic lipid nanocarriers might be utilized for drug targeting. Cancer cells, for instance, are known for an altered lipid membrane composition and increased membrane fluidity [154]. Cell membranes from patients with malignant lymphoma and leukemia showing a higher unsaturated lipid content and less cholesterol in cellular membranes, displayed a higher membrane fluidity as compared with those of normal lymphocytes [155–157]. These differences in membrane fluidity of normal and tumor cells were already exploited in the early 2000s for targeted delivery of anticancer drugs using liposomes as vehicle that selectively fuse with cancer cells. In vitro, a good correlation between membrane fluidity on various types of tumor cells and the anti-cancer effect of liposomes was found [158]. Liposomes, for instance, were shown to fuse with human hepatocellular carcinoma cells but to a much lower extent with normal hepatocytes [159]. In another study, liposomes primarily made of fluid-state lipids targeted tumor cells, while liposomes made of rigid-state lipids targeted normal cells [15]. Although the concept of targeting cells that display a higher membrane fluidity with fusogenic lipid nanocarriers seems convincing, so far, such delivery systems have not been subject of clinical trials. It is therefore on us to enhance the efficacy of these systems. In particular, their selectivity for cancer cells and fusogenic properties need to be further improved. The combination of high membrane fluidity as target with another tumor specific target such as the overexpression of certain enzymes in the same lipid nanocarrier might be a promising strategy. So-called dual targeting nanocarriers have already shown encouraging results [160–162]. So far, however, high membrane fluidity of tumor cells has not been combined with other targeting approaches.

Furthermore, cubosomes—formed when amphiphilic lipids such as monoolein self-assemble into cubic phases in excess water—are promising fusogenic nanocarriers. Their potential lies in their highly negatively curved membranes, which facilitate fusion with cellular membranes. In an in vitro study, cubosome uptake by small intestinal cells via membrane fusion was observed within just 30 s [163]. Additionally, cubosomes possess an exceptionally high surface area-to-volume ratio, enabling substantial drug-loading capacity within their inner liquid crystalline structures [164]. They have already been employed to deliver large therapeutic molecules, such as nucleotides and antibodies, to exosomes. Notably, their fusogenic properties allowed nearly 100 % loading of challenging macromolecules like mRNA and immunoglobulin G into exosomes within only 10 min of mixing drug-loaded cubosomes with exosome solutions [165]. The enhanced bioavailability of various drugs incorporated into cubosomes [166–168] can, at least to some extent, be attributed to their fusogenic properties.

Another interesting concept might be the introduction of charge converting properties to fusogenic lipid nanocarriers. On the one hand a cationic surface charge is essential to trigger the fusion process. On the other hand, however, cationic nanocarriers do not reach their target, as they are already immobilized by ionic interactions to anionic substructures found in blood, extracellular matrix or off-target cells. This so-called ‘polycation dilemma of drug delivery’ can be overcome by charge converting delivery systems that display an anionic charge on their way to target cells converting their charge to positive values directly at the target cell [169]. Such targeted charge conversions can be triggered by pH, enzymes or under reducing conditions [170]. Until now, this approach has been used mainly to trigger endocytosis, but the concept should also show potential in case of fusogenic lipid nanocarriers. At least a proof-of-concept is already available in form of fusogenic liposomes containing inverse-phosphocholine lipids for triggered anionic-to-cationic charge conversion. The cleavage of phosphate groups on the surface of these liposomes by phosphatases that are overexpressed in various tumors and inflamed tissue [171] caused a loss in anionic charges and a raised impact of the remaining cationic charges. Although

the shift in zeta potential was poor and did not even reach positive values, an enhanced fusion with target cells could be shown [172]. Meanwhile there are much more potent charge converting systems available that effectively convert their zeta potential from negative to true positive values [170] and that can be easily transferred to fusogenic lipid nanocarriers. In view of this progress, the concept deserves further investigations.

Another interesting concept that also deserves further investigations is calcium mediated fusogenic lipid nanocarriers. Calcium ions interact with negatively charged components of the cellular membrane such as phosphatidylserine and phosphatidic acid. The ionic crosslinking of these negatively charged membrane substructures leads to aggregation and subsequently fusion of adjacent membranes [173]. Although the mechanism is known since the 1970s and numerous studies have been undertaken to harness this fusion mechanism for the design of fusogenic nanocarriers [174–176], there are still not any convincing in vivo data available. To trigger fusion processes calcium concentrations $\geq$5 mM seem to be necessary [177]. In in vitro experiments calcium concentrations can be adjusted ad libitum but raising the calcium concentration to that demanded level in target tissues in vivo where lipid nanocarriers shall fuse is another story. On top of that, the raise in local calcium concentration has to be synchronized with the arrival of lipid nanocarriers at the target membrane. Nonetheless, when fusion is intended to take place directly at the injection site, the development of such delivery systems seems feasible as fusogenic nanocarriers can be injected with a highly concentrated calcium solution. This approach might end up in sound product developments.

7. Conclusion

Biological fusion events including fertilization, exocytosis, plasma membrane repair or viral infections prove the potential of this mechanism to overcome cellular barriers. By copying nature, we have learned to design lipid nanocarriers that can fuse with target cells and release their payload into them. Addressing key parameters such as curvature, fluidity and surface charge and additional decoration with fusogenic (poly)peptides resulted in delivery systems that show high potential to fuse with target cells. Most of these systems, however, exhibit this potential just in vitro when they are directly brought into contact with target cells and in comparison to other technologies there are only a very few convincing in vivo studies available. The efficacy of fusogenic nanocarriers is obviously not sufficiently high to show its potential also in more complex and challenging systems such as organisms. In order to enhance their efficacy, we have to improve our analytical tools to generate more detailed insights in the fusion behavior of different formulations (i), to take the in vivo situation in the design of our delivery systems more carefully into consideration (ii) and to combine the fusogenic approach with other approaches (iii). Since the number of hydrophilic macromolecular drugs such as therapeutic peptides and proteins as well as nucleic-based drugs is strongly raising and most of them have their target inside cells, the demand for vehicles that can shuttle their payload across membranes into cells is increasing. This industrial demand will likely also accelerate the development of fusogenic nanocarriers. Furthermore, we should be aware of the great toolbox of registered inactive ingredients that is available for the design of such delivery systems providing high flexibility in formulation design. Taken all, fusogenic lipid nanocarriers have the potential to shape the future landscape of intracellular drug delivery. It is on us, to take advantage of it.

CRediT authorship contribution statement

Gennaro Balenzano: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation. **Numan Eczacioglu:** Writing – review & editing, Writing – original draft, Visualization, Investigation. **Nunzio Denora:** Writing – review & editing, Visualization, Supervision. **Andreas Bernkop-Schnürch:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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