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Nanoprecipitation- and nanoemulsification-based systems for efficient nucleic acid delivery

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Nanosystems are essential for ensuring protection, stability and efficient delivery of nucleic acids (NAs) across biological barriers, unlocking a new era of medicinal potentials. Messenger RNA (mRNA), small interfering RNA (siRNA), microRNA (miRNA) and plasmid DNA (pDNA), among others, have notably been explored as therapeutics in combination with nanoscale systems in applications such as vaccination and gene silencing. Over the years, a plethora of colloidal systems has been developed through diverse formulation approaches with nanoprecipitation and nanoemulsification emerging as efficient and scalable strategies able to tune and control the physicochemical properties of the produced nanocarriers. In the present review we highlight recent progress in nanoprecipitation and nanoemulsification approaches for the development of nanosystems with genetic cargo, discussing their principles, formulation parameters, and their application across different carrier systems including lipid nanoparticles (LNPs), liposomes, polymeric nanoparticles (PNPs), hybrid structures and nanoemulsions (NEs). Composition, encapsulation efficiency (EE), and biological performance along with scalability and clinical translation are examined as key factors shaping next-generation platforms for NA therapeutics, offering opportunities to refine delivery efficiency, reduce toxicity, and ultimately increasing patient access to advanced therapies.

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Introduction

Deciphering the human genome has enabled the development of nucleic acid (NA)- based strategies for the treatment and prevention of conditions ranging from global threats to rare genetic disorders. Messenger RNA (mRNA), small interfering RNA (siRNA), circular RNA (circRNA), double-stranded RNA (dsRNA), antisense oligonucleotides (ASOs) and plasmid DNA (pDNA), among others, can elicit or silence specific biological responses. Their rapid production and modifiable nature enable targeting to even undruggable targets [1*]. Yet limitations persist in achieving intracellular delivery and stability due to degradation by extracellular enzymes, non-specific distribution, and recognition by the immune system. Despite the challenges, the potential of NA-based therapies is evident from approved products (DAWNZERA™, mResvia®) and completed or ongoing clinical trials (NCT06672237, NCT06961006), which demonstrate feasibility not only due to chemical modifications of NAs (e.g. pseudouridine modifications [2]), but also through the use of colloidal carriers, which are equally important.

Transitioning from immunogenic viral vectors with risk of host genome integration, colloidal nanocarriers have emerged as promising alternative and have evolved considerably since their first use in the '70s. NA encapsulation in colloidal systems protects against degradation, enhances cellular uptake, enables controlled and targeted release, and can facilitate endosomal escape while promoting controlled immune activation and minimizing off-target effects [3]. Furthermore, they maintain structural stability, a perquisite for cargo protection, long shelf-life and along with their scalability, ensure translation into widespread therapeutic use [4]. These benefits have driven the development of diverse nanoparticles (NPs) tailored for NA delivery involving quantum dots [5], polymeric NPs [6], lipid-based NPs [7], solid lipid NPs [8], liposomes [9], nanoemulsions (NEs) [10] and others. Of these, lipid nanoparticles (LNPs) are the most extensively studied due to their success during the COVID-19 pandemic [11]. These NPs can be developed through various methods (i.e. thin film hydration, sonication, solvent evaporation) [12] with nanoprecipitation and nanoemulsification to be the most applied approaches

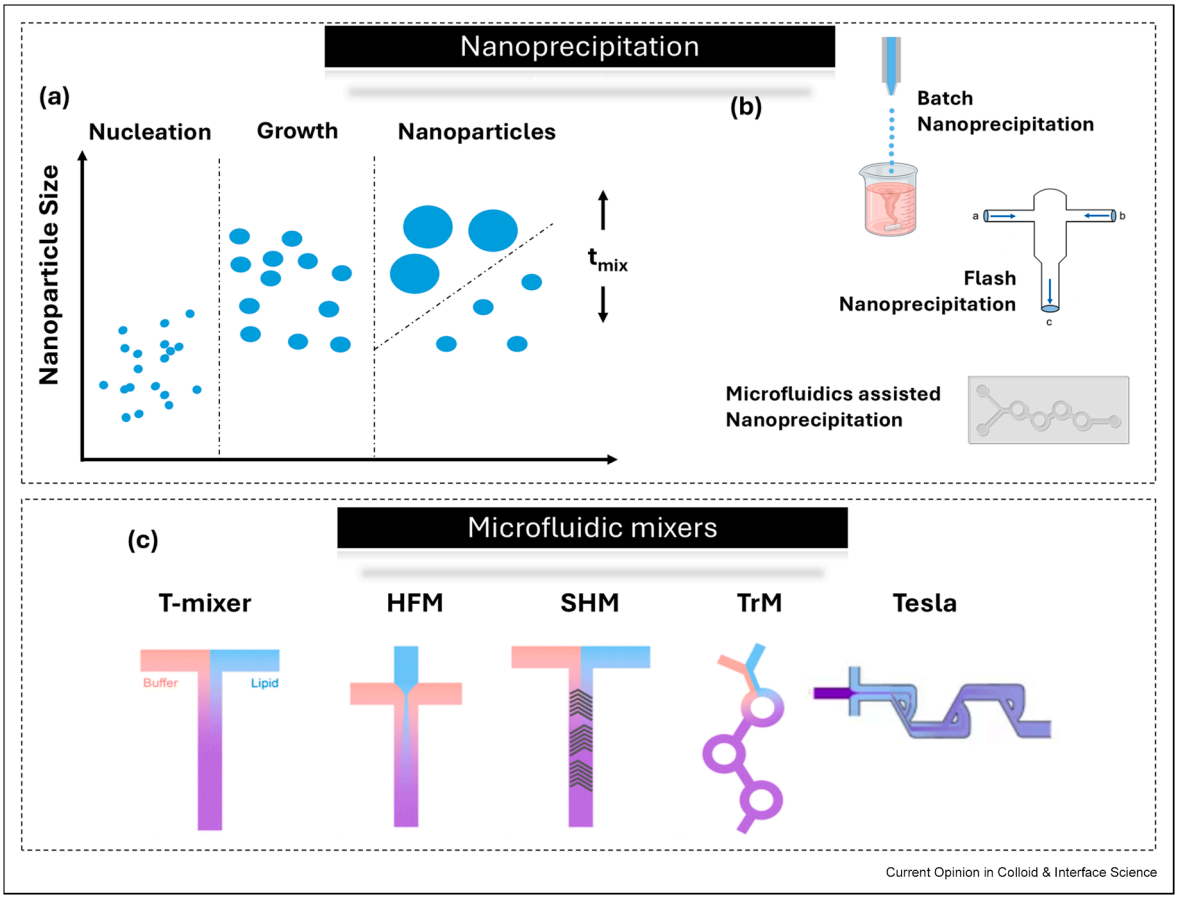
for NA-NPs. In this review, we examine nanoprecipitation and nanoemulsification as key methods for producing NPs for NA delivery while we discuss their principles, advantages, and current applications, highlighting their potential in driving future advances in NA therapeutics and clinical translation.

Principles of nanoprecipitation

Nanoprecipitation (aka solvent displacement) stands out as an approach for developing NPs offering simplicity, reproducibility, and scalability while protecting the encapsulated payload. The seminal work by Fessi in 1989 [13], introduced the concept of transition from miscibility to immiscibility applied to the development of polymeric nanocapsules. Essentially, nanoprecipitation involves dissolving a hydrophobic solute, the NP-forming material (e.g., polymers, lipids), in a water-miscible organic solvent. This solution is then rapidly introduced into a larger volume of an aqueous non-solvent phase (NP- forming material is immiscible in it), where the compound intended for encapsulation is dissolved. Upon rapid mixing, the polar organic solvent diffuses quickly into the aqueous phase, causing a significant decrease in the solubility of the NP-forming material and subsequently for minimizing thermodynamically unfavorable interactions with the surrounding aqueous environment, self-assembles or aggregates [14]. A specific case of nanoprecipitation occurring in multicomponent liquid systems is known as the ouzo effect [14]. The simplest case to observe is a ternary liquid system, such as the ouzo example (an alcoholic beverage common in Greece and other Mediterranean countries). When water is added to a dilute binary solution of a solute whose solubility in water is very small (e.g., anise oil) in a solvent (e.g., ethanol), most of the solute will rapidly come out of solution. For certain proportions of solute, solvent, and water, a relatively stable dispersion of very small solute droplets is formed. This process of creating metastable liquid–liquid dispersions is termed "the ouzo effect".

Nanoprecipitation, as well as the ouzo effect, occurs when solutions are rapidly brought sufficiently into the metastable region (a stage where the Gibbs free energy

Figure 1



Schematic representation of (**a**) basic principle of nanoprecipitation (adapted with permission from Ref. [26]) (**b**) common nanoprecipitation techniques and (**c**) main microfluidic-assisted approaches for nanoparticle (NP) preparation (left to right) T-junction mixer, hydrodynamic flow focusing (HFF), staggered herringbone mixer (SHM), toroidal mixer (TrM), and Tesla mixer.

is not minimized but the phase separation is limited by large kinetic barriers) by the addition of water. When the solubility of some of the solutes decreases more rapidly than linearly with increasing water concentration, the solution can become supersaturated in these components. If the supersaturation is large, nuclei form spontaneously from small local fluctuations in concentration of solute molecules. This process is known as homogeneous nucleation. The nuclei have a larger than average concentration of solute. Thus, their formation causes a depletion of solute near each nucleus. As a result, further nucleation occurs only in the depleted regions, as far as possible from existing nuclei. Nucleation ends when no regions remain with a high supersaturation. Nucleation must be separated by the next stage (particle growth) for low polydispersity while at the final step solvent removal (dialysis or evaporation) or additives presence stabilizes the NPs [15] (Figure 1). The result of this process (which takes place on the millisecond or faster time scale) is a spatially, fairly uniform dispersion of very small liquid droplets (in the case of the ouzo-effect) or polymeric or LNPs (in the case of nanoprecipitation) suspended in the continuous liquid phase [14].

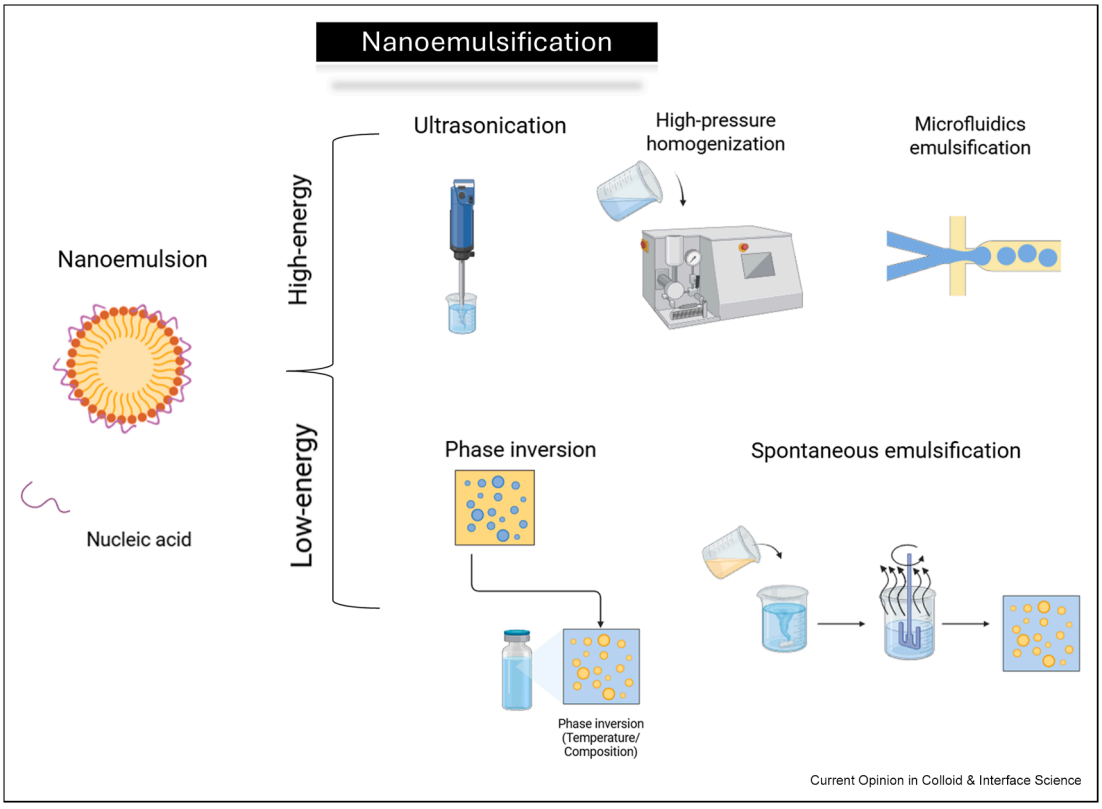
Nanoprecipitation approaches can be categorized by mixing mechanism in (a) batch (BNP) (b) flash (FNP) and (c) microfluidic-assisted nanoprecipitation (MNP) (Figure 1). BNP typically involves the one-step addition of the solvent into the larger non-solvent volume, dropwise or by pipette mixing [16], primarily employed for initial lab scale experiments. Although simple, it may face challenges regarding reproducibility, scalability, and encapsulation efficiency (EE) for NAs due to the poor control over mixing. The mixing time (t_{mix}) in this approach is generally in the order of seconds to minutes, with the pipette mixing leading to faster but less uniform results. For shorter t_{mix}, FNP relies on rapid (<3 ms), turbulent mixing within a confined environment (e.g., confined impingement jets- CIJ and multi-inlet vortex mixer-MIVM) first described by Johnson and Prud'homme [17]. It generates supersaturation and nucleation in milliseconds, leading to the formation of smaller, more uniform and of higher EE NPs compared to BNP [18]. Although FNP is scalable and reproducible, its dependence on specialized equipment, exhibiting reduced adaptability for multi-component formulations. Addressing the increasing demand for scalable and high-throughput NPs production in nanomedicine, MNP offers mixing control by manipulating the fluid flow through adaptable geometries, channel flow rates, offering scalability through parallelization while minimizing human introduced variability. The most representative microfluidic systems in NA field are passive (i.e. by special microchannel geometries) including T-junctions [19], hydrodynamic flow focusing (HFF) [20*], staggered herringbone mixing (SHMs) [21], baffle [22], toroidal (TrM, bifurcating mixers) [23**] and Tesla [24] mixers (Figure 1). SHM and TrM are commercially available, used in the NanoAssemblr™ Benchtop and NxGen™ T-junction (NanoAssemblr™ Ignite) platforms, respectively. Active systems are also available and facilitate external energy sources such as acoustic waves [25], however their use is limited by added complexity and cost.

Principles of nanoemulsification

Nanoemulsification is a core technique in colloid science for producing emulsions at the nanometric scale, providing a versatile alternative to nanoprecipitation in the fabrication of nanosystems designed to deliver NAs and other bioactive agents. The process involves the dispersion of two immiscible liquids in the presence of surfactants, an organic and an aqueous phase, into kinetically stable nanodroplets through energy input, which can be introduced either through high- [27] or low-energy methods [28]. The underlying theoretical framework integrates thermodynamics, kinetics, and surfactant science to explain droplet formation, stabilization, and long-term behavior.

In high-energy methods such as high-pressure homogenization, microfluidization, and ultrasonication, intense shear, turbulence, and cavitation forces disrupt the fluid–fluid interface once the applied stress exceeds the Laplace pressure, generating fine, kinetically stable droplets (Figure 2). High-pressure homogenization, where emulsions are forced through narrow orifices under pressures typically between 200 and 1500 bars, is widely employed for its ability to precisely regulate droplet dimensions and produce stable, monodisperse NEs. In microfluidization for NEs preparation, a coarse emulsion is forced through fixed geometry microchannels at pressures up to ~275 MPa, generating intense shear, turbulence, and cavitation to produce fine, stable NEs. Ultrasonication, in contrast, provides cavitation forces that break the coarse emulsion into smaller nanodroplets. These approaches, even though effective and scalable, involve significant energy input and may expose NAs to shear and thermal stress [29]. On the other hand, low-energy methods exploit the physicochemical behavior of surfactants and co-surfactants to induce phase inversion or spontaneous emulsification, relying on interfacial instabilities and thermodynamic transitions rather than external mechanical energy. According to Jafari and McClements [30], the droplet size distribution and stability in nanoemulsification are theoretically described by the Kolmogorov–Hinze theory and interfacial thermodynamics, while long-term stability arises from surfactant-mediated steric and electrostatic barriers as described by the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory, which accounts for the balance between van der Waals attraction and electrostatic repulsion.

Figure 2



High and low-energy methods for the preparation of nanoemulsions (NEs).

Spontaneous emulsification and phase inversion temperature are most applied in pharmaceutical formulations. Both approaches impose fundamental limits on droplet size: in mechanical methods, size reduction may be constrained by surfactant availability, while in thermodynamic methods, the ability of the surfactants to lower the interfacial tension governs the final dimensions. Despite their advantages in mild processing and scalability, low-energy techniques require careful optimization due to surfactant-related toxicity and complex inter-droplet interactions.

Stability also remains a critical challenge, while the presence of surfactants imposes more problems such as extensive in vitro toxicity studies and more to dive in. The complex colloidal phenomena such as the ones dictating instability, interdroplet interactions are of significant importance for the study of NA-NE systems not only for their inclusion as drug delivery systems but also for their use as templates for the development of organic and inorganic NPs [31].

Nanocarriers for NA delivery

Liposomes and lipoplexes

Liposomes are colloidal, artificial, phospholipid vesicles, where the internal aqueous core separates from the surrounding aqueous environment, enabling simultaneously the encapsulation of varying polarity molecules in their distinct domains. Based on their morphology, they are categorized into multivesicular (MVVs), multilamellar (MLVs), oligolamellar (OLVs), and unilamellar vesicles (ULVs), while size provides additional level of classification [32]. Since Felgner's 1987 study showed that cationic N-[1-(2,3-Dioleyloxy) propyl]-N,N,N-trimethylammonium (DOTMA) liposomes could improve transfection of pDNA [33], liposomes have emerged as nanosystems for NA delivery.

Several methods are employed for liposome preparation, including thin film hydration [34], surfactant removal [35], ethanol injection method (EIM) [36], and microfluidics [37], with the latter two related to nanoprecipitation. EIM is considered a straightforward way to develop liposomes that avoid specialized equipment, lengthy hydration steps, and solvent residues. Lipids are dissolved in ethanol or methanol and rapidly injected into an aqueous NA solution, where phospholipids precipitate and then reassemble to form mainly planar bilayers that finally close after the evaporation of the organic solvent. Composition wise, these liposomes include cationic lipid(s) (Table 1) (20–60 mol%) such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [36], DOTMA [38,39], dimethyldioctadecylammonium bromide

Table 1

Representative nanosystems employed for nucleic acid (NA) delivery. The table summarizes common compositional compounds, NA cargo, particle size, polydispersity index (PDI), and encapsulation efficiency (EE). Commonly used preparation techniques are listed along with potential therapeutic applications as mentioned in the literature.

Composition	Cargo	Size (nm)	PDI	EE (%)	Application	Techniques
Lipid nanoparticles (LNPs)						
Ionizable, cationic lipids SM-102, MC3, ALC-0315, DODAP, DODMA, C12-200, or analogs Phospholipid structural lipid DSPC, DPPC, DOPE, DOPC Cholesterol PEG-lipids DMG-PEG2000, DSPE-PEG2000, C14-PEG2000, or PEG-DMG Optional targeting/functional lipid Anisamide, mannose, DOPE analogs	mRNA saRNA siRNA pDNA Combinations CRISPR-related	Typical diameter: 70–150 nm for most LNPs Observed ranges depending on formulation: 65–200 nm	0.07–0.2, indicating generally narrow size distributions Observed ranges up to 0.3, depending on formulation, cargo, and preparation method	Typical EE: >90 % Observed ranges: 70–100 %, depending on cargo type, formulation, and method	Liver-targeted gene expression and protein delivery [65,72] Vaccination and immune system modulation [50**] Lung-targeted therapy (anti-inflammatory, anti-tumor, endothelial protein expression) [58**,59] Reproductive system therapy [60,61] Anti-infective applications (CRISPR-Cas delivery against pathogens) [124] Proof-of-concept/ methodological studies (bioluminescence, post-encapsulation stability) [52]	**Microfluidic-based techniques** T-junction mixers [76,78] Microfluidic ultrasound cavitation-assisted nanoprecipitation [88] NanoAssemblr ™ platforms [50**,79] Baffle mixer [85] 3D printed devices [83*] **Bulk mixing techniques** Vortex mixer [12] Rapid pipette mixing **Flash nanoprecipitation** CIJ mixer [51,90]
Liposomes/Lipoplexes						
Cationic/ionizable lipids DOTAP, DOTMA, DDAB, DC-1-16, DC-6-14, TC1-12 Phospholipids DSPC, DPPC, DOPE, DOPC, Egg-PC, EDOPC Cholesterol and derivatives PEGylated lipids DSPE-PEG2000, DSPE-PEG-Maleimide, C16-PEG2000, PEG2000PE Additional coatings/functionalization: HSA	mRNA siRNA miRNA Other NAs Poly I:C (dsRNA),aptamers	Typical diameter: 100–136 nm Observed ranges: 85-280	Typical PDI: ~0.2 Observed range: Up to <0.36	Typical efficiency: 85–99 % Efficiency can increase with higher N/P ratio Some reports: 70–80 %	Tumor imaging and therapy [41] Lung-targeted therapy (fibrosis, anti-miR delivery) [42] Liver-targeted protein expression [40] Spleen-targeted immune modulation [38] Brain-targeted therapy (Parkinson's disease model) [44]	**Microfluidic-based techniques** SHM [21] Y-shaped microfluidic chip [38] Flow-focusing [41] and micropillar-mixing channels [20*] **Bulk mixing techniques** Ethanol injection [36,39][(±sonication) [42]
Nanoemulsions						
Ionizable/cationic lipids C12–200, DOTAP, SM-102 Neutral/helper lipids/phospholipids DOPE, DSPC, Soybean lecithin Oils/lipid phases Vitamin E, Captex 800 NF, Soybean oil, Oleic acid, MCT	mRNA: siRNA miRNA: circRNA	Typical size range: 60–150 nm Observed extremes: <100 nm–178 nm	0.09–0.24	Typical efficiency: 75–100 % Most of the cases refer to surface complexation	Brain delivery (CNS, glioblastoma, Parkinson's disease, depressive like behaviors) [114,117] Intranasal mRNA vaccination [115] Treatment of sepsis-induced	**Microfluidic-based techniques** NanoAssemblr™ platforms [114] **Bulk mixing techniques** Bulk mixing [115]nanoemulsions followed by electrostatic complexation of RNA

(*continued on next page*)

Table 1. (*continued*)

Composition	Cargo	Size (nm)	PDI	EE (%)	Application	Techniques
Surfactants/PEGylated lipids: DMG-PEG2000, Tween 80, DSG-PEG2000, Kolliphor HS15 Other components: 1,8-Cineole, perillyl alcohol					cardiovascular injury [116**] Tumor therapy via siRNA targeting metastasis-related proteins (e.g., Twist1) [10]	**Nanoemulsification** High-speed shearing and conjugation [116**]+ microfluidics [10] Emulsion injection into buffer followed by probe sonication [117*]
Other systems **Polymeric nanoparticles** **Polymer-lipid HNPs;** **Lipid-polymer-lipid systems; core-shell with inorganic components; cell membrane-coated HNPs; nanoparticle-in-nanoparticle** Polymers PLGA, mPEG–PCL, polyhydroxyalkanoate, poly (acrylic acid), cationic oligomers Lipids DOTAP, DC-Chol, DDAB, DPPC, DOPE, cholesterol, ALC-0315 Surfactants/stabilizers: PEI, lipid–peptide conjugates Inorganic/functional additives: Calcium carbonate, gold nanoparticles, Cell-derived materials: Immune cell membranes, cancer cell membranes, extracellular vesicle membranes	siRNA mRNA pDNA dsRNA circRNA	Sizes typically ≈100–180 nm, depending on core and shell composition	PDI varies with method: Nanoemulsification tends to give higher PDI	siRNA: Up to 85–100 % mRNA: Near-complete encapsulation in microfluidic pDNA: Enhanced via lipopolyplex formation	Cancer therapy (breast cancer [98], glioblastoma [99], hepatocellular carcinoma [106], colorectal carcinoma) Immunotherapy/ immunostimulant delivery Brain-targeted delivery Co-delivery of drugs and nucleic acids for enhanced therapeutic effect	**One-step approaches** Ethanol injection [103], BNP [98], TrM microfluidics [100,105], emulsification [104] FNP [101] **Two-step approaches** Core formation → shell application (electrostatic adsorption, co-extrusion, microfluidics) [106] **Hybrid strategies** Combination of microfluidics + sonication [109]or co-extrusion for cell membrane-coated HNPs [107]

Abbreviations: LNPs, lipid nanoparticles; **SM-102**, heptadecan-9-yl 8-((2-hydroxyethyl) (6-oxo-6-(undecyloxy)hexyl)amino)octanoate; **MC3**, (6*Z*,9*Z*,28*Z*,31*Z*)-Heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate; **ALC-0315**, ((4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate); **DODAP**, 1,2-dioleoyl-3-dimethylammonium-propane; **DODMA**, 1,2-dioleyloxy-N,N-dimethyl-3-aminopropane; **C12–200**, 1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl) (2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol); **DSPC**, 1,2-distearoyl-sn-glycero-3-phosphocholine; **DPPC**, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; **DOPE**, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; **DOPC**, 1,2-dioleoyl-sn-glycero-3-phosphocholine; **DMG-PEG2000**, 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000; **DSPE-PEG2000**, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]; **HSA**, human serum albumin; **mRNA**, messenger RNA; **saRNA**, self-amplifying RNA; **siRNA**, small interfering RNA; **miRNA**, microRNA; **circRNA**, circular RNA; **pDNA**, plasmid DNA; **dsRNA**, double-stranded RNA; **Poly I:C**, polyinosinic-polycytidylic acid; **N/P ratio**, nitrogen-to-phosphate ratio; **CNS**, central nervous system; **FNP**, flash nanoprecipitation; **BNP**, batch nanoprecipitation; **TrM**, Toroidal mixer; **PEG**, polyethylene glycol; **MCT**, medium-chain triglycerides; **HNPs**, hybrid nanoparticles; **PEI**, polyethyleneimine.

(DDAB) [36,40] for NA complexation, facilitating the transfection, while also affecting in vivo targeting (DOTAP targets liver/spleen; DDAB lungs [40]). Neutral phospholipids (mainly zwitterionic) include 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) [36,39],2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) [36] which are commonly used to supplement the systems affecting their efficacy (i.e. DOPE instead of DOPC leads in improved gene knockout independently of the cationic lipids [36]) as they are important for membrane fusion and endosomal escape. Cholesterol ensures bilayer stability, while polyethylene glycol (PEG) lipids (1−2%) provide stealth properties. Longer PEG chains can exntend blood circulation time, but de-PEGylation is needed for efficient transfection with shorter chains to favor fusion [36]. NAs are either incorporated during liposome formation [41] or electrostatically associated with pre-formed cationic liposomes to form lipoplexes [39,42]. pDNA [43], siRNA [36], mRNA [40] and synthetic dsRNA [41] have been successfully incorporated through EIM while co-encapsulation of NAs-drugs is also possible. For instance, siRNA and a monoamine oxidase-B inhibitor incorporated within a single liposome, yielded EE 92.35 % and 78.66 %, respectively [44].

Microfluidics produce liposomes with consistent physicochemical properties and increased EE [45] while continuous processing allows increased bench-to-industrial reproducibility, overcoming the lack of mixing control during EIM. For example, HFF [41] and SHM [37] have been employed resulting in monodisperse liposomes while the lipid composition used remained consistent with EIM. Parameters such as lipid nature and concentration, total flow rate (TFR, the sum of the volumetric flow rates of all input channel) and flow rate ratio (FRR, the ratio of the flow rates between the different fluids) are important. Stiffer membranes require more energy to form vesicles, leading to larger liposomes, i.e., liposomes of unsaturated DOPC (lower bending modulus) were smaller (∼50 nm) and more stable than those of saturated 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) or 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) (∼100 nm) [37*,46]. Lipid concentration of the organic solution (1−30 mM [38]) is the factor that governs the kinetics of the nucleation and growth of the nanoprecipitates with higher lipid composition leading to larger particle size [45]. Below the suitable TFR (usually 1−8) [41,45,46], mixing efficiency can decrease to the extent where the t_{mix} is above the timescale of nanoprecipitation, resulting in liposomes of larger sizes, while FRR controls the nucleation of nanoprecipitates and the formation of liposomes. General operational FRRs for SHM range typically from 1 to 5 [45], compared to 2 to 10 for HFF [20*]. The organic phase-usually ethanol or occasionally methanol [46]-should solubilize the lipids while miscible with the aqueous phase (e.g. citrate buffer [46,47]) and compatible with microfluidic devices. The increased positively charged amine groups to negatively charged phosphate groups (N/P) ratio in case of siRNA loading increased the complexation (88 %) and as a result the size [45]. New studies have evolved microfluidic fabrication as well as the introduction of short, single stranded NAs (aptamers), demonstrating a cost-effective fabrication of high-precision 3D-printed for the HFF (liposome preparation) and micropillar-mixing channels (aptamer modification) advancing targeted drug delivery approaches along with photoacoustic imaging [20*].

Lipid nanoparticles (LNPs)

As cationic lipids raised issues regarding toxicity, ionizable lipids (IL), neutral in physiological pH and positively charged in acidic environment, led to the formation of LNPs [48]. The FDA approval of Onpattro® and COVID-19 vaccines established LNPs in medicinal forefront for NA delivery as evidenced by the increasing number of active clinical trials (i.e., NCT06686654, NCT05969041, NCT03739931). LNPs have a lipid core instead of the aqueous one in liposomes, while apart from the IL they share the same ingredients with liposomes (IL, cholesterol, helper lipid, PEG-conjugate) [49] (Table 1). Interestingly, the ingredients' essentiality was challenged by the successful addition of an immunopotentiating lipid instead of cholesterol for enhanced immunogenic properties [50**] while other than PEG polymers have been used for their stealth properties [51**] important to reduce side effects from this polymer.

In general, for LNPs preparation, a lipid ethanolic solution is mixed with an acidic NA solution inducing electrostatic interactions between the negatively charged NA and positively charged lipids, forming inverted micelles with lipids surrounding the NA. Ethanol falls below its critical concentration for lipid solubilization, triggering precipitation and self-assembly of lipid particles via EIM, MNP and FNP. Post-encapsulation, though not widely used, has recently gained attention as an attractive strategy to reduce NA losses and costs for "on-demand" mRNA loading, ranging from straightforward mixing and MNP [52] to NA−bridged LNP-to-LNP fusion triggered by pH shifts [53]. This approach remains underexplored, with factors such as lipid membrane rigidity, surface charge, and polymer density likely dictating its success. Cargo type strongly affects LNPs as siRNA is compactly encapsulated, while larger mRNAs form blebbed morphologies while polyuridylic acid lead to stability issues [54,55]. In addition, mixing strategies affect differently NA incorporation, as mRNA achieved high EE (94−100 %) regardless of the mixing method, whereas siRNA showed higher efficiency (73 %) when using HFF on a lab-scale (TFR 5 mL/h) [56*]. LNPs with liquid crystalline inverse hexagonal lipid structures (e.g. by increased DOPE) actively encapsulate and show higher

transfection efficiency than LNPs with lamellar structures [57], whereas no such improvement was observed for mRNA [58**].

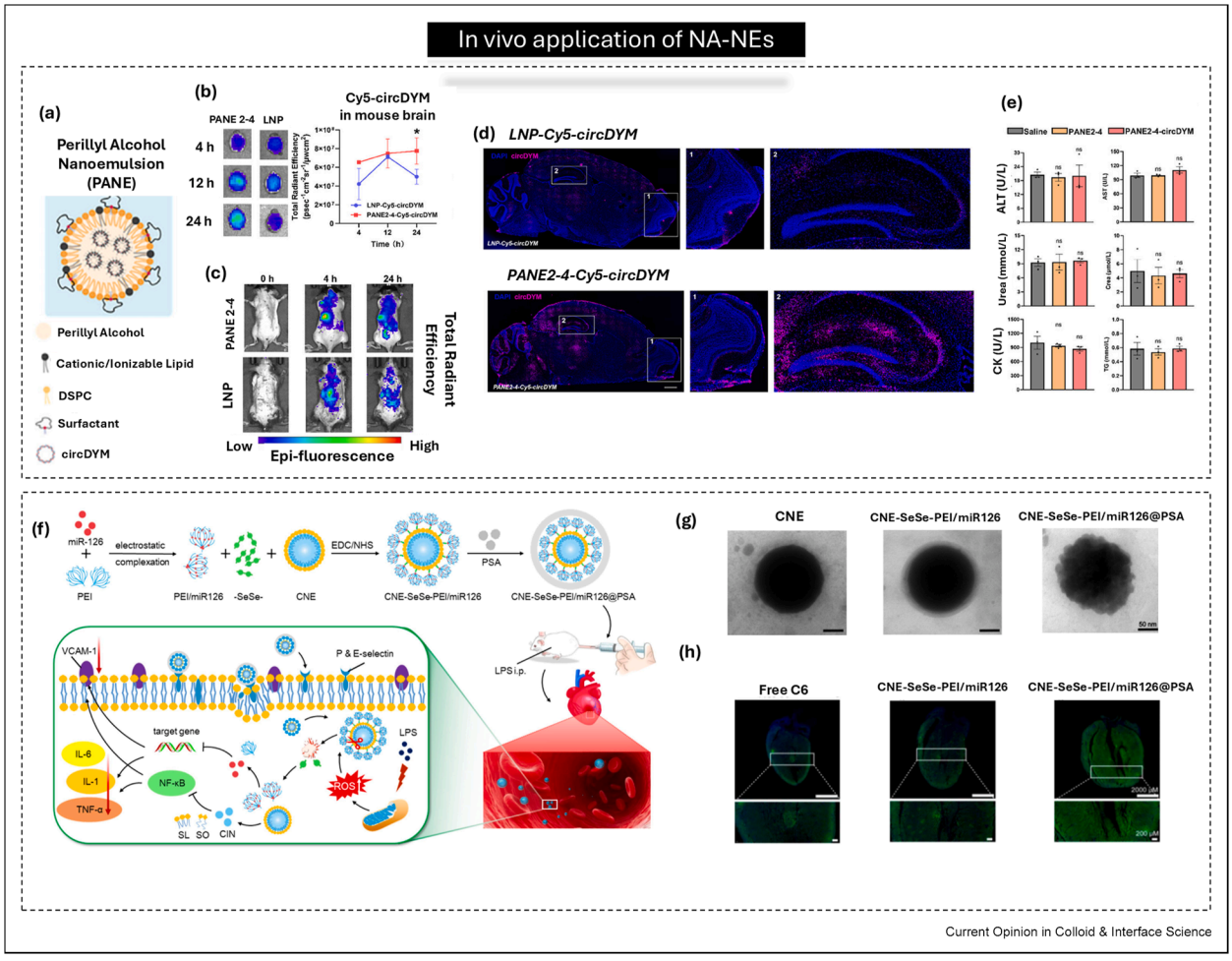
BNP has been used for encapsulation of mRNA [59], single-stranded self-amplifying RNA (ssaRNA) [60], DNA barcodes [61], pDNA [62] as well as for the co-delivery of drugs/NAs [63]. Adjusting parameters such as stirring rate, organic solvent/antisolvent ratio, and lipid ratios can result in different sizes and EE. The organic solvent/antisolvent ratio is usually 1:3−1:4 [59,61,64,65]. Lipid concentrations (1−20 mg/mL) in the organic phase, N/P ratio (commonly 3−6 for mRNA), injection rate in case of robotic mixer (speed 500 µL/s) [66], temperature (20−80 °C) and buffer nature further modulate NP characteristics. Slower mixing often leads to larger, polydisperse particles and reduced EE [60,66,67]. BNP is thus mainly used for early screening, while microfluidics supports higher-volume production with comparable physicochemical properties [65] (although some face challenges with this transition [68**]). Notably, detailed morphological comparison remains scarce, despite the clear influence of mixing on LNP structure (e.g., lamellarity [69]) and function [23**,60].

The need for reproducible NA-loaded LNPs with controlled physicochemical properties, and scalability has driven the field towards MNP approaches for smaller LNPs of diverse compositions and cargos including mRNA [70,71], siRNA [72,73], ASOs [54], and others [69,74]. MNP has outperformed BNP upon careful parameter modulation [68**], which could alter size, structure and even organ tropism [68**]. TFR, FRR, pH and ionic strength of the buffer along with N/P ratio and exact composition play all fundamental role in the formation of the final LNPs and their efficiency. For instance, using 50 mM citrate buffer tends to produce smaller LNPs compared to the ones prepared with 100- and 300- mM due to reduced bleb structures [75]. The bleb structure is intended to be correlated with mRNA protection and efficacy though with controversial results [75,76]. Changing lipid ratios revealed architectures from highly immunogenic emulsion-like LNPs to liposome-like structures affecting strongly structure, immune responses, extrahepatic accumulation and altered protein corona composition [19,77**]. Following LNP formation, the final evaporation of the organic phase is a critical step to ensure solvent removal, facilitate lipid fusion, and enhance NP stability and performance such as tangential-flow filtration which not only removes ethanol but also facilitates particle fusion and stability by controlling pH changes [78].

Highly controlled LNP production is achieved using SHM geometries, such as the commercial NanoAssemblr™ (up to 21 mL/min) and the high-throughput TrM NxGEN™ technology (1−200 mL/min) [23**,79,80] which provide low shear stress to preserve NA cargo integrity. SHM typically produces LNPs <100 nm with polydispersity index (PDI) ≈0.2, strongly influenced by FRR (commonly 3:1−6:1 aqueous/ethanol [54,73]) and TFR (3−21 mL/min [69,74,81]). The N/P ratio is usually around 3 for siRNA [56*,58**] and up to 10 for mRNA [72], while IL and PEGylated lipids affect both the resulting particle size and EE [81]. A N/P 10 ratio for IL: siRNA resulted in high EE (>87 %), narrow PDI, and a size of 65 nm, leading to a dense core rather than the classic bleb structure [72]. Conversely, increasing the PEGylated lipid content can lead to smaller particle sizes due to a higher surface area-to-volume ratio [73]. Initial lipid concentrations (typically 10−15 mM) also affect outcomes as lower concentrations (∼2 mM) can work for cost-efficient screenings, though in vitro and in vivo data are limited [54]. A comparison of SHM and HFF showed both small, high-EE particles, but only SHM-derived mRNA LNPs retained in vitro activity, whereas siRNA LNPs were effective regardless of method [72].

Advancements for improved scalability, size control, throughput, and reproducibility involve the development of new devices (Figure 3). The circular asymmetric split-and-recombination (CA/SAR) system, for example, combines laminar diffusion with Dean vortex mixing [82], offering superior control over NPs formation. The 3D-printed Omnidirectional Sheath-flow Enabled Microfluidics (OSEM) device, combining a novel 4-way HFF region with a SHM for enhanced mixing precision achieving production rate 60 ml/min [83*], while membrane micromixing [84] (i.e. the organic phase passes through microporous membranes into a circulating aqueous phase) enabling high flow rates up to 500 mL/min, in case of mRNA. The invasive lipid nanoparticle production (iLiNP) platform (baffle mixers) employs piling and numbering-up strategies, maintaining precise size control at high TFR (20 mL/min per device), offering autoclave compatibility, and supporting large-scale vaccine production (∼34,000 doses/hour) [85]. A silicon scalable LNP generation (SCALAR) has achieved 17 L/h production by incorporating up to 256 chip units of glass/silicone chips [86**]. However, the significant challenge in continuous microfluidic LNP production is fouling which over time leads to reduced particle homogeneity and EE, potentially causing device failure. To mitigate fouling, microchannels enlargement [83*], tethered lubricant oil layers [87] and ultrasound application [88] have been employed with positive results. The latter, through the application of 10 W ultrasound, reduced shear stress on mRNA, improved EE by ∼20 %, preserved RNA integrity at levels comparable to BNT162b2 vaccine, and prevented clogging for up to 1.5 h of continuous production. The dual vortex 3D-printed micromixer prevents clogging with round channels and enables

Figure 3



Advancements in microfluidic mixers for the preparation of nucleic acid (NA)-lipid nanoparticles (LNPs). (**a**) On-chip de novo production of mRNA-LNPs. Fluorescence microscopy image of a Tesla mixer-based microfluidic device used for LNP generation with fluorescein solution, and schematic representation of on-chip RNA–LNP generation (adapted from Ref. [24]) (**b**) Schematic of aptamer-modified liposome synthesis using a flow-focusing and micropillar-mixing channel microfluidic device, with corresponding images of the fabricated chips (adapted with permission from Ref. [20*]) (**c**) Side view of a 3D-printed Omnidirectional Sheath-flow Enabled Microfluidics (OSEM) device, featuring a four-way sheath flow channel followed by a downstream SHM for LNP production; photograph demonstrating four-way sheath flow using water and ethanol (red dye), with corresponding cryo-Transmission Electron Microscopy (cryo-TEM) images (scale bar 100 nm) (adapted with permission from Ref. [83*]) (**d**) Schematic and image of the silicon scalable LNP generation (SCALAR) chip, showing parallelized configurations (10× and 256×) with fluorescent images illustrating mixing. Cryo-TEM of luciferase mRNA–LNPs produced by the SCALAR 10 × chip (scale bar 100 nm) (adapted from Ref. [86**]).

efficient LNP production matching commercial systems after 12 h of continuous use while maintaining in vivo efficacy [89]. These results are very promising for rapid translation to the market, although further structural characterization and in vivo testing should be applied.

FNP has also proven effective in generating NPs with tunable properties in a single step, as it enables rapid mixing through confined impingement in turbulent flow, producing NPs with narrow size distribution at high throughput as proved by Pfizer-BioNTech COVID-19 vaccine. FNP with a CIJ and μMIVMs mixer was used to prepare a wide cargo loaded LNPs siRNA, mRNA presenting high EE (above 80 %), up to 140 nm sizes with 0.15 PDI [90].

The LNP field continues its rapid evolution with notable advaces such as integrating all steps from mRNA synthesis to encapsulation in a single microfluidic device, with yields matching or surpassing conventional methods [24]. Despite such progress, future work must address comprehensive structural characterization, in vivo efficacy, toxicity, and scalability to fully realize the potential of these platforms.

Polymeric nanoparticles (PNPs)

Polymers are considered the primary materials in the field of drug delivery, as they possess the advantage of flexible and versatile chemical structures. Hence, polymeric nanoparticles (PNPs) have been developed to enhance the stability of RNAs and facilitate their delivery. PNPs can achieve drug transfer with minimal degradation, continual and steady release, and better drug activity through gradual degrading polymer matrix or shell, which makes them suitable candidates for NA delivery [91]. PNPs can be prepared by several methods, with the most common being nanoprecipitation, emulsification, and electrospray. To evaluate the different methods, Roshan et al. [92] prepared poly (lactic-co-glycolic acid) (PLGA) polymer carriers to encapsulate curcumin (CUR) as model drug, using two different methods, namely electrospray and nanoprecipitation, intending to compare the efficacy of the resulted PNPs. The group valued the electrospray method as superior to nanoprecipitation, as it led to smaller particle size, lower PDI and drug loading percentage. Their findings also showed higher EE (93.2 %) and loading capacity (7.2 %) for electrosprayed PLGA particles and slower CUR release rate, proving electrospray a better technique for PLGA fabrication.

Cationic polymers such as poly (β-amino ester) (PBAE) (a class of biodegradable polymers containing ester bonds in their main chain) are extensively investigated as carriers of siRNA. These NPs are fabricated in two steps, starting with the construction of a polymer platform consisted by PBAEs (PBAE precursor polymers), followed by the termination with hydrophilic or charged amines. Following this methodology, Chen and co-workers [93] managed to develop 14 precursor polymers and tested the resulting alkyl chain-capped PBAEs towards the delivery of siRNA in HeLa-Luc cells. To further improve their carriers, the group proceeded to the co-formulation of PBAE carriers with helper lipids, leading to the reduced size and surface charges of the polyplex NPs with siRNA. Consequently, they managed to decrease the cytotoxicity and enhance siRNA delivery efficacy.

Amphiphilic poly (lactic acid)-block-poly (carboxybetaine) (PLA-b-PCB) zwitterionic polymers have also been used for RNA release [94]. After binding PLA-b-PCB polymers with acid-responsive hemiacetal pendant groups, they produced polymer-lipid nanocarriers that could encapsulate both siRNA and mRNA with high efficiency. They found that acid-responsive release of RNA from the PLNPs was necessary for increased efficacy. They also characterized their carriers as superior to LNPs as far as siRNA release was concerned. In these mixed PNPs, the ionizable MC3 lipid was found to be responsible for endosomal escape.

A crucial issue to consider when drug delivery is concerned is the safety of the developed formulation. In this respect, not only should the materials used (e.g., polymer) be biocompatible, but the resulting formulation should also be free of traces of residual organic solvents. This is crucial since PNPs are almost exclusively obtained by formulation processes such as nanoprecipitation, emulsion-solvent evaporation etc. that involve the use of organic solvents (e.g., chloroform, dimethylformamide or methanol). Some of them are difficult to remove completely from formulations, therefore a toxicity issue is risen for the development of pharmaceuticals. As an answer to this problem, Guerassimoff et al. [95] developed a methodology for polymer pro-drug NPs preparation that does not involve organic solvents (an all-aqueous formulation process). They prepared gemcitabine-conjugated poly (2-hydroxyethyl acrylate)-block-poly (2-hydroxyethyl acrylate-co-2-(hydroxy methyl) thio-propyl) (PHEA) and dual-drug NPs containing both this copolymer and doxorubicin by dissolving the diblock copolymer in water at 4 °C, either in the presence or absence of doxorubicin hydrochloride at a defined copolymer-to-drug ratio, and then the copolymer solution was added dropwise into a glass vial prefilled with water containing Pluronic F68 under constant stirring at 60 °C. This is a temperature controlled all-aqueous nanoprecipitation process that allowed them to formulate PNPs without the use of organic solvent, avoiding the remaining traces of toxic compounds.

Even though PNPs are among the most extensively developed NPs, their translational obstacles remain mainly because at physiological pH the systems, opposed to LNPs, are positively charged, which can lead to cellular damage and of target effects. However, their diversity and customizability are big advantages for NA delivery.

Hybrid systems (HNPs)

Hybrid NPs (HNPs) constitute a category of nanocarriers that integrate the structural and functional benefits of diverse materials, making them particularly promising in different biomedical contexts. By combining organic nanomaterials with inorganic components or cells and cell-derived materials, these systems can improve pharmacokinetics [96], encapsulation, and stability of genetic payloads, while boosting biological performance [97]. HNPs can be engineered in configurations such as: (a) a polymeric core surrounded by a lipid shell, (b) a lipid NP enclosed within a polymeric shell or (c) a NP encased in a cell-derived lipid-based environment, with many other designs continuously emerging. The synthesis of HNPs generally follows one-step or two-step approaches, influencing the final structure, stability, and performance of the system. One-step approaches include thin-film hydration, nanoprecipitation, and emulsification-based techniques. Despite the relatively mild processing conditions and low energy requirements, emulsification methods can lead to high PDI, while one-step

nanoprecipitation, even though being more straightforward, can suffer from batch-to-batch variability. Two-step approaches usually begin by forming the core particle and the chosen shell is applied in a subsequent step, commonly through electrostatic adsorption, co-extrusion, or microfluidics.

Polymer-lipid HNPs is a common configuration in NA delivery. In such systems, polymers could be combined with lipids through nanoprecipitation. For example, methoxy polyethylene glycol−polycaprolactone (mPEG−PCL) has been combined with the cationic lipid dimethyldioctadecylammonium bromide (DDAB) to formulate siRNA-loaded NPs (upon complexation) for breast cancer therapy [98] while PLGA with DOTAP or 3β-[N-(N′,N′-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) for glioblastoma siRNA treatment [99]. N/P ratio affected strongly the siRNA complexation (N/P $\geq$ 10 for 85 % binding), while the lipid nature -depending on the cell target-affected the final efficiency [99]. Flow conditions significantly influence Eudragit core−lipid hybrid LNPs for pDNA delivery prepared via one-step microfluidics with a DC-cholesterol shell. Increasing the aqueous-to-organic phase FRR reduced particle size, while also doubling the z-potential at 3:1 FRR, reflecting altered component distribution. Transmission Electron Microscopy (TEM) showed that BNP produced systems had full lipid coverage of the polymer core, whereas microfluidics at 3:1−5:1 FRR led from damaged to disrupted structures. This disruption correlated with higher cell toxicity, likely due to polymer exposure and increased z-potential. These findings highlight the importance of understanding LNP structure, as it can impact size, surface charge, and biological effects [100]. In another configuration, pH-responsive amorphous calcium carbonate NPs stabilized with poly (acrylic acid) developed through FNP and coated with polyethyleneimine (PEI) and siRNA benefiting from the stabilizing and condensing effects of PEI [101]. In case of lipopolyplexes, polymers-pDNA NPs inserted in a microfluidic device with prepared DPPC/DOPE/Chol liposomes and leading to 3-times higher transfection, revealed that the polymer plays crucial role in the structure of the system [102]. Even more sophisticated systems have been developed, such as chemical electron transfer (CET)-based lipopolyplexes where a lipid shell embedding a CET donor (oxalate derivative) and a hydrophilic core containing gold nanoparticles, siRNA, and a cationic oligomer. In tumor cells this disrupts endosomes, releasing siRNA into the cytoplasm to achieve tumor gene silencing and mitotic arrest [103]. Recently, to transition from synthetic polymers, biosynthetic ones based on medium-chain-length polyhydroxyalkanoates are used for increased biocompatibility. In this case, NPs size was highly sensitive to manufacturing conditions with nanoprecipitation approach to produce smaller and more uniform particles compared to nanoemulsification [104]. However, an important feature is that these studies lack strong biological evaluation raising questions about their applicability and side effects. Liposomes developed through SHM device encapsulating siRNA were rapidly mixed with PLGA organic phase facilitating the spontaneous self-assembly of PLGA around the liposome-siRNA, which rigidity aids tumor uptake-a parameter not widely taken into account-and stability in mucus [105]. Interestingly, microfluidic-enabled serial assembly (MESA) provides a sequential route to hybrid nanoparticles. A cationic core is first formed from sorafenib and a lipid−peptide conjugate, then siRNA is adsorbed under slowed flow, and finally a lipid shell is added. This process achieves near-complete encapsulation of both drug and siRNA and enables effective co-delivery for hepatocellular carcinoma therapy [106].

Cell membrane coated NPs is another promising strategy, typically achieved through a two-step process, for efficient targeting and uptake by leveraging the affinity of cellular membranes along with the structural integrity of their core. Usually as a first step EIM is facilitated to complex IL and phospholipids with the cargo mRNA [107] or siRNA [108] forming LNPs, followed by co-extrusion/sonication with membranes from immune [107] to cancer cells [108] and extracellular vesicle (EV) membranes. Tello et al. developed EVs−LNP hybrids using ALC-0315 LNPs and EVs derived from tendon mesenchymal cells via a one-step microfluidic process combined with sonication, achieving successful cytoplasmic delivery of collagen type I mRNA. Despite a ∼20 % reduction in EE and cellular uptake, likely due to the hybrids' increased negative charge, this approach demonstrated the feasibility of EV−LNP fusion for NA delivery [109]. The nanoparticle-in-nanoparticle (NIN) HNP encapsulated dsRNA immunostimulant and a lipid-modified cyclodextrin containing a small molecule inhibitor. These ∼120 nm HNPs are formed via MNP with classic parameters such as FRR 3:1. A critical innovation for this system's success is the lipophilic modification of the cyclodextrin, which facilitates its incorporation into the LNP's internal structure. This dual-payload system demonstrated potent efficacy, leading to the complete eradication of murine colorectal carcinoma tumors [110**].

HNPs represent a dynamic and rapidly evolving field with significant potential for advancing therapeutic and diagnostic strategies. Further research into optimizing synthesis methods, exploring novel material combinations, and understanding their complex biological interactions will be crucial for their successful clinical translation.

Nanoemulsions (NEs)

NEs are colloidal dispersions consisting of nanoscale droplets (typically 50−300 nm in diameter) of one

immiscible fluid dispersed within another. Typically, the two fluids are oil and water, but other immiscible liquids can also be emulsified upon surfactant presence. Their optical appearance-ranging from transparent to translucent or milky-depends primarily on droplet size. NEs can be either water-in-oil (w/o) or oil-in-water (o/w). Preparation methods are categorized into high-energy techniques, such as high-pressure homogenization, microfluidization, and ultrasonication, and low-energy approaches including spontaneous emulsification and phase inversion emulsification techniques. They offer a versatile platform for the delivery of drugs and other bioactive compounds via multiple routes of administration [111,112].

While conventional o/w NEs offer excellent solubilization and bioavailability for hydrophobic drugs, the development of cationic NEs extends their utility into the field of NA therapeutics, where positive surface charges enable efficient complexation and cellular uptake of negatively charged macromolecules. Cationic NEs (CNEs) have an overall positive surface charge consisting of commercially available oils, phospholipid(s), cationic lipid(s), and non-ionic surfactant(s). The lipids enable electrostatic bonding with NAs while the N/P critically influencing release kinetics and loading efficiency. RNA molecules such as siRNA or mRNA have been complexed or encapsulated within CNEs, systems that could also facilitate endosomal escape allowing RNA release into the cytoplasm. Encapsulating part of the cationic lipids within the oil core may help reduce toxicity, while a deeper understanding of the system's structural organization is essential for enhancing transfection efficiency and achieving prolonged therapeutic effects [113].

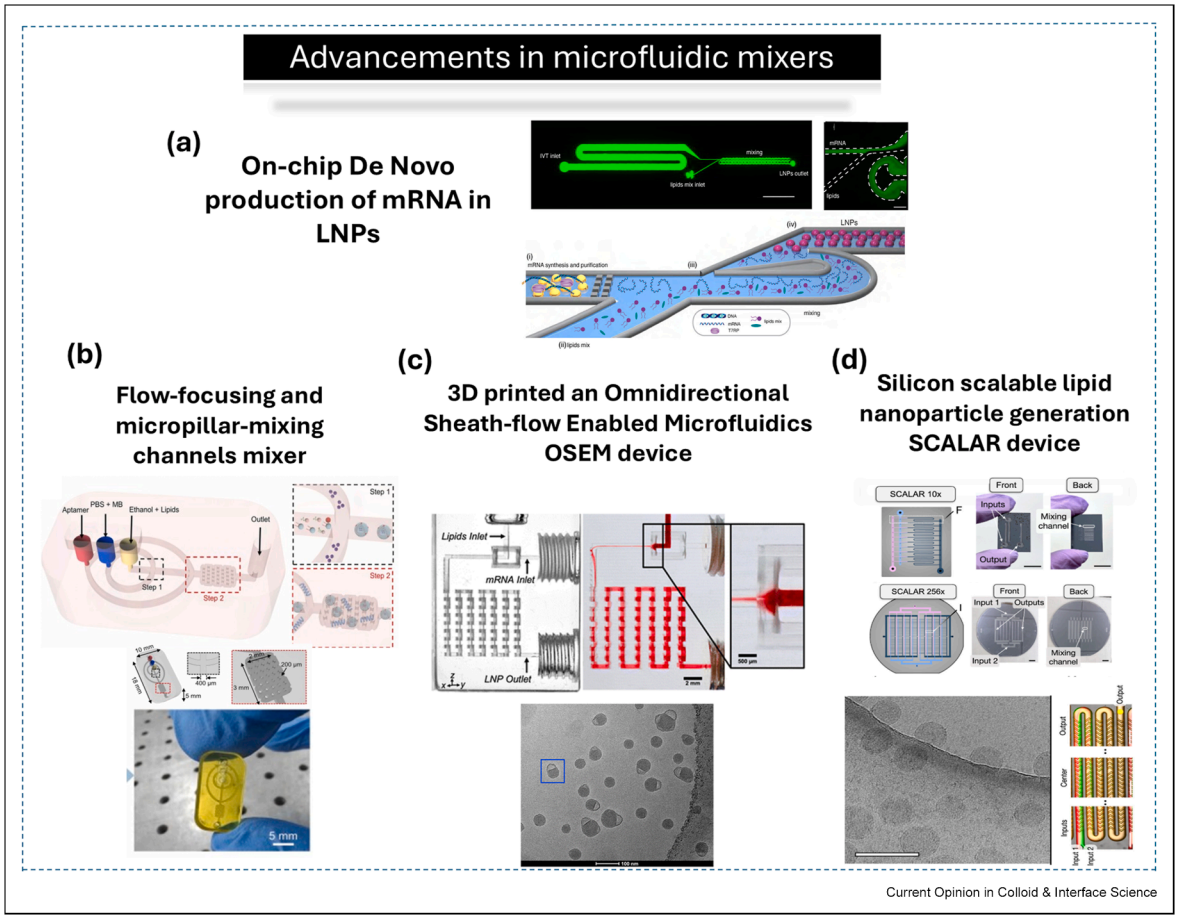
Despite their effectiveness in NA delivery, CNEs raise concerns over toxicity and off-target effects due to cationic surfactants and lipids which can disrupt membranes, induce oxidative stress, and trigger immune responses. Ionizable NEs (iNEs) represent a strategic advancement, combining the benefits of NEs with ionizable lipids. This approach offers improved safety profiles and precision delivery, particularly for RNA-based therapeutics. A work by Borrajo et al. presents iNEs as a promising platform for targeted RNA delivery in the brain, with potential applications in treating CNS disorders such as glioblastoma and Parkinson's disease [114]. The formulated, through nanoprecipitation, iNEs, composed of C12−200, DOPE, Vitamin E, and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol (DMG)-PEG, exhibited a particle size under 100 nm, a neutral surface charge, and high RNA loading capacity (80−90 %). These properties translated into excellent biocompatibility and robust transfection efficiency across multiple cellular models, including neurons, astrocytes, and microglia. Similarly, iNEs were developed for nasal vaccination with the aim of protecting and delivering mRNA. As a result, a new generation of NEs incorporating a combination of an ionizable and a cationic lipid were developed and optimized as mRNA vaccine candidates for intranasal administration. These nanocarriers were composed of a combination of cationic or IL (DOTAP and C12-200, respectively), a helper lipid (DOPE), an oil (Vitamin E or Captex800 NF), and a non-ionic surfactant (Tween® 80). This NE exhibited a size of 120 nm and a highly positive surface charge (+50 mV). Upon mRNA complexation, PDI reduction was noted, attributed to the enhanced stabilization of the nanosystem through the electrostatic complexation on the surface. Interestingly, two different approaches were used for the preparation of mRNA loaded NEs: bulk mixing of cationic blank NEs followed by the electrostatic complexation of mRNA onto the surface; or microfluidic production of NEs in the presence of mRNA, in a single step, using a microfluidic mixer system [115].

Several modified NE platforms have been explored for NA delivery. For example, a study conducted by Wang et al. presents a polysialic acid (PSA)-modified, Reactive Oxygen Species (ROS)-responsive NE designed for the targeted co-delivery of 1,8-Cineole and miRNA-126 to alleviate septic cardiac dysfunction. PEI, a well-established biodegradable cationic polymer and widely used nonviral gene carrier, was employed to deliver miR-126 due to its strong gene condensation capacity. The nanosystem comprises a 1,8-Cineole-loaded NEs conjugated with a PEI/miR-126 complex via a ROS-sensitive linker. PSA functionalization on the surface enhances endothelial targeting through specific interactions with selectins, enabling efficient and selective delivery as proved through cardiovascular injury-induced in vivo murine model [116**] (Figure 4).

In another study, Gao et al. developed perillyl alcohol NEs for the targeted delivery of circDYM to the brain, resulting in the alleviation of depressive-like behaviors. The formulations comprising of perillyl alcohol: ionizable/cationic lipids: DSPC: Tween 80 to achieve efficient circRNA incorporation and delivery. circRNA has recently gained attention as a promising therapeutic platform for various diseases, including cancer and infectious conditions. These NEs, developed through probe sonication, exhibited excellent RNA loading capacity (>89 %) and maintained stability over extended storage periods [117]. Interestingly, circDYM was encapsulated by the cationic lipid inside the PANEs which was recognized by TEM and the NEs surpassed the efficiency of LNPs in the absence of perillyl alcohol (Figure 4).

In a study of Padilla et al., o/w NEs were developed for delivering siRNA targeting Twist1, a key driver of tumor metastasis in various cancers. The NE employs Medium Chain Triglycerides (MCT)-an FDA-approved oil-as the

Figure 4



Nanoemulsions (NEs) in Nucleic Acid (NA) Delivery. (**a**) The structure of NEs composed of perillyl alcohol (PANE), cationic or ionizable lipids, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and surfactants for circDYM encapsulation delivery (**b**) Fluorescent imaging and quantification of Cy5-circDYM in mouse brains at 4, 12, and 24 h after intranasal administration of lipid nanoparticles (LNP-Cy5-circDYM) and PANE2-4-Cy5-circDYM (**c**) Representative in vivo fluorescence imaging and quantification of Cy5-circDYM in mouse bodies after intranasal administration of LNP-Cy5-circDYM and PANE2-4-Cy5-circDYM (**d**) Representative fluorescence images and quantification of Cy5-circDYM fluorescence in mouse olfactory bulb. (**e**) Plasma levels of biochemical markers for toxicity from mice treated with saline and NEs (adapted from Ref. [117*]). (**f**) Schematical illustration of the preparation of CNE-SeSe-PEI/miR126@PSA NPs and its targeting and synergistic anti-inflammatory mechanism (**g**) TEM images of PSA@CNE-SeSe-PEI/miR126, CNE-SeSe-PEI/miR126, and CNE. (**h**) autofluorescence on the cryo-sectioned slides of heart compared with free C6 (adapted upon permission from Ref. [116**]).

hydrophobic core. To enhance siRNA solubility in MCT, the siRNA is complexed with DOTAP, forming a hydrophobic ion pair. This MCT/siRNA-DOTAP complex is then formulated into a stable NE with an average particle size of 140 nm, exhibiting long-term stability for up to 195 days. In an in vivo murine tumor model, the NE achieved a 46 % reduction in Twist1 mRNA levels within 48 h post-administration, demonstrating its potential as an effective siRNA delivery platform for anti-metastatic cancer therapy [10]. These modified NE systems exemplify the growing innovation in nano-based RNA delivery, with formulations increasingly tailored for biological specificity, responsiveness, and safety in complex disease settings.

Comprehensive characterization of nanoparticles

The translation of NA-NPs into clinic necessitates thorough characterization, from structure and stability analysis to activity and safety assessment. Apart from large-scale production with consistent therapeutic outcomes, their characterization will contribute to the development of research databases and standardized reporting systems for every step from bench to market.

Regardless of the system type, structural and physicochemical characterization is foundational for the development and understanding of NA-NPs. Essential parameter is the particle size which can govern

biodistribution, cellular uptake and consequently targeting and immune responses. For example, smaller LNPs in vaccine delivery are less immunogenic in mice compared to larger ones (>100 nm) [118]. Hydrodynamic diameter (ζ-average) is primarily assessed by Dynamic Light Scattering (DLS) (usually back scatter at 173°) or Multi-Angle DLS (MADLS) along with PDI which characterizes the homogeneity of particle population [38]. DLS provides an intensity-weighted average size from a single angle, while MADLS follows a multi-angle detection approach offering higher resolution. In DLS particles are assumed spherical while the measurement focuses on hydrodynamic diameter only, possessing limitations. Both size and PDI can be measured over longer storage periods to demonstrate the structural stability of the system [36,66,79], however, it should be highlighted that structural stability has been more extensively studied than NA integrity and efficiency. Along with DLS, Nano Tracking Analysis (NTA) [73,75,77**,109] provides particle concentration data, visualizes individual particle motion (through Brownian motion analysis), and enables better discrimination between populations close in size, thus allowing the study of systems with higher polydispersity. NanoFCM is also employed for single-particle characterization and concentration measurements [53] while other techniques such as analytical ultracentrifugation (AUC) [55], have also been used, though they are not common practice. The zeta potential (ζ-potential) surface charge, is another important factor for colloidal stability and interaction with the environment and is commonly measured with Electrophoretic Light Scattering (ELS) [55], most of the time implemented in the same equipment of DLS. Other techniques, such as Laser Doppler Velocimetry (LDV) [119] and Phase Analysis Light Scattering (PALS) [81] have also been used for this purpose. Reported ζ-potentials range from positive values (∼+20-+30 mV for polyplexes [93] or high N/P lipoplexes [42]) to slightly negative/near-neutral values (≈>-10 mV for optimized mRNA LNPs at physiological pH [81]) with positive surface charge to generally enhance cellular uptake and endosomal escape but increases serum opsonization, toxicity, and rapid clearance.

Structural and compositional analysis is equally critical, especially as DLS method assumes spherical structure. Microscopy techniques such as Scanning Electron Microscopy (SEM) [41,92], TEM [41], and Atomic Force Microscopy (AFM) [105] provide information on morphology, surface topography, and microstructure elucidating important features for the NP circulation time, cellular interactions and others. Cryo-TEM [42,50**,97], particularly with tomography capabilities [57], enables direct visualization of lamellarity, internal organization, and overall integrity of the systems along with cargo. In case of PEG-lipids, cryo-EM helped to understand that they further modify the core organization of ASO-loaded LNPs, which govern further its gene silencing properties [120]. In addition, DOPE-containing LNPs likely achieved higher transfection efficiency through enhanced endosomal escape, attributed to the formation of inverted hexagonal phases opposed to DSPC lamellar, predominantly round morphologies as cryo-TEM revealed [58**]. Furthermore, Small-Angle X-ray Scattering (SAXS) [39,54,55]and Small-Angle Neutron Scattering (SANS) provide data for structure, shape, size and nanoparticles' interactions with NA. For instance, SAXS and Nucleic Magnetic Resonance (NMR) study on cationic DOTMA/DOPE mRNA lipoplexes revealed that initial ULVs (empty liposomes) reorganized into multilamellar stacks of repeating lipid bilayers where mRNA inserted into the hydrophilic spaces between them, forming a condensed NP [39], highlighting the necessity of advanced methods for structural studies. Thermal and compositional analyses such as Differential Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA), and Nuclear Magnetic Resonance (NMR) [78,95] provide insights into component interactions. The apparent acid dissociation constant (pKa) determines the ionization behavior and surface charge of the nanoparticles, with values around 6−7 typically optimizing endosomal escape, usually measured using the fluorescence-based TNS assay [50**,53,55,68**].

EE remains a defining parameter for the performance of LNPs and related systems. Commonly, RNA content is quantified using assays such as Quant-iT^{TM} RiboGreen^{TM} for mRNA [24,38,53], siRNA [44] and anti-mir-21 [42], Quant-iT^{TM} PicoGreen^{R} for dsDNA [51,69] and Quant-it^{TM} Oligreen^{TM} for ssDNA [54]. Following disruption of LNPs with surfactants, the percentage of encapsulated NA is calculated relative to the unencapsulated fraction, calculated before the detergent application. It has to be mentioned though that recent studies indicate that these calculations can overestimate true loading, often exceeding 85 %. When EE is instead calculated relative to the original input RNA efficiencies frequently fall below 50 %, highlighting the need for standardized reporting [121,122]. In some cases, EE is determined by ultrafiltration, physically separating free, unencapsulated from formulated complexes, followed by spectrophotometric quantification of the free fraction to calculate the incorporated percentage [41,51]. The Qubit fluorometer is also employed with detergent-mediated LNP disruption [23**,24] while integrity of NA can be assessed with gel electrophoresis [24,98] or LC-UV-HRMS [82].

Moving beyond structure, the in vitro and in vivo potency determines the functional performance of the LNP as a delivery vehicle. In vitro assays quantify the expression of the target protein, cellular uptake and trafficking are quantified via Flow Cytometry [58**], while Confocal Light Scanning Microscopy (CLSM)

tracks intracellular localization to confirm endosomal escape [58**,60,75]. The metric of LNP activity is the gene expression, which is quantified using highly sensitive assays such as the Luciferase Reporter Assay, ELISA for secreted proteins, or qPCR for gene knockdown. Biodistribution is an essential functional parameter, mapped through in vivo studies utilizing fluorescently or radiolabeled NPs to confirm tissue-specific accumulation and the systemic circulation profile toward the intended target organ, imaging techniques such as in vivo fluorescence with the use of deep-tissue NIR dyes [42] or bioluminescence imaging -especially luciferase expressing mRNA- [50,53,97] and photoacoustic imaging [123] provide real-time quantitative and spatial data.

Toxicity is assessed in vitro using dose-dependent viability assays, using colorimetric tetrazolium reduction assays such as MTT/MTS [38] and CCK-8 [37*,69], while hemolysis assay [73,116] assess vascular toxicity. General animal safety, or in vivo tolerability, is monitored by tracking body weight, behavior, and assessing major organ damage via histopathology post-administration. A critical component of safety is understanding immunogenicity and inflammation which is usually measured via ELISA to quantify the release of pro-inflammatory cytokines (e.g., IL-6 [55], TNF-a [40]) from immune cells, or to detect the presence of antibodies following repeat dosing [50**].

Collectively, the integration of physicochemical, structural, and biological characterization is fundamental for the rational development of nanocarriers and for bridging formulation design with therapeutic translation.

Conclusions and future perspectives

Recent progress in continuous manufacturing, particularly through microfluidics, has strengthened nanosystems' potential for clinical translation by addressing some of the long-standing challenges in reproducibility and scale-up.

However, their transition from proof-of-concept studies to clinical implementation isn't without significant hurdles. For example, the presence of surfactants during nanoemulsification along with the exposed NA or the residual organic solvents in nanoprecipitation approaches may induce unwanted side effects and potential immune activation. Beyond the formulation-specific issues, in the literature there is no consensus on characterization standards for the NA-encapsulated systems. Parameters such as EE are often reported inconsistently or even omitted. Establishing standardized protocols and databases would be beneficial to navigate in the world of NA-encapsulated nanocarriers to greatly improve reproducibility and facilitate regulatory evaluation.

Looking ahead, scaling up microfluidic platforms to meet industrial demands poses considerable technical and economic challenges but innovations such as end-to-end microfluidic devices (from mRNA production to LNPs development) for acceleration of personalized medicine are of great importance. At the same time, artificial intelligence (AI), and machine learning (ML) [96] will play a transformative role, enabling predictive modeling for formulation design stability and process optimization, particularly relevant when dealing with complex biomolecules, where minor changes in formulation can profoundly impact stability and bioactivity.

The plethora of systems in literature for many purposes calls for a shift toward rational design, guided by clear mechanistic understanding and application-driven objectives with nanoprecipitation- and nanoemulsification-based carriers evolving into reliable platforms for drug delivery, therapeutics, and clinical diagnostics.

Credit author statement

Aristotelis Xenakis: Writing – original draft, Writing – review & editing, **Vassiliki Papadimitriou:** Writing – original draft, Writing – review & editing, **Maria Zoumpanioti:** Writing – original draft, Writing – review & editing, **Evgenia Mitsou:** Conceptualization, Writing – original draft, Writing – review & editing.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cocis.2026.102005.

Data availability

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

References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

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