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DEVELOPMENT AND EVALUATION OF LIPID–POLYMER HYBRID NANOPARTICLES FOR TARGETED SIRNA DELIVERY IN CANCER THERAPY

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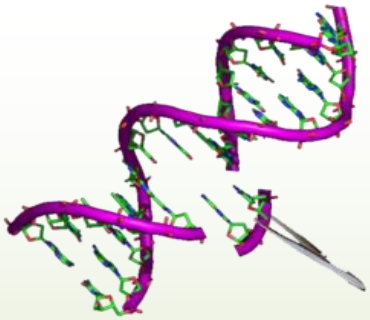


INTRODUCTION

1.1 Overview of Gene Therapy

Definition and Principles

Gene therapy is a therapeutic approach that involves the introduction, removal, or modification of genetic material within a patient's cells to treat or prevent disease. The fundamental principle is to correct or regulate defective genes responsible for disease pathogenesis rather than merely treating symptoms. The concept was first formally proposed in the 1970s and later advanced through recombinant DNA technology and molecular biology innovations Gene therapy works by delivering nucleic acids—such as DNA or RNA—into target cells using viral or non-viral vectors. The therapeutic gene must reach the appropriate intracellular compartment (nucleus or cytoplasm) to exert its function. Efficient gene delivery requires overcoming multiple biological barriers, including enzymatic degradation, immune recognition, cellular uptake, and endosomal escape.

Two primary delivery systems are used

Viral vectors (e.g., adenovirus, lentivirus)

Non-viral vectors (e.g., liposomes, polymeric nanoparticles, lipid–polymer hybrid nanoparticles).

Non-viral systems have gained importance due to improved safety profiles and reduced immunogenicity compared to viral vectors.

Types of Gene Therapy

Gene therapy can be broadly classified into three main types.

1. Gene Replacement Therapy

Gene replacement therapy involves introducing a functional copy of a defective or missing gene into a patient's cells. This approach is primarily used for monogenic disorders such as cystic fibrosis and severe combined immunodeficiency (SCID). The therapeutic gene is typically delivered using viral vectors to ensure stable expression (Naldini, 2015, p. 351–360).

A landmark example includes the FDA approval of Luxturna for inherited retinal disease, demonstrating the clinical success of gene replacement strategies.

2. Gene Silencing (siRNA-Based Therapy)

Gene silencing involves suppressing the expression of disease-causing genes rather than replacing them. Small interfering RNA (siRNA) mediates post-transcriptional gene silencing through the RNA interference (RNAi) pathway.

The RNAi mechanism was discovered by Andrew Fire and Craig Mello in 1998, for which they received the Nobel Prize. siRNA molecules bind complementary mRNA sequences, leading to their degradation via the RNA-induced silencing complex (RISC) (Fire et al., 1998, p. 806–811).

Mechanism

siRNA enters cytoplasm.
Incorporated into RISC.
Target mRNA is cleaved
Protein translation is inhibited.

siRNA therapy is highly specific and is particularly promising in cancer therapy where oncogenes can be selectively silenced. However, siRNA is unstable in circulation and requires protective carriers such as lipid nanoparticles or lipid–polymer hybrid nanoparticles for effective delivery (Whitehead et al., 2009, p. 1298–1308).

3. CRISPR-Based Gene Editing

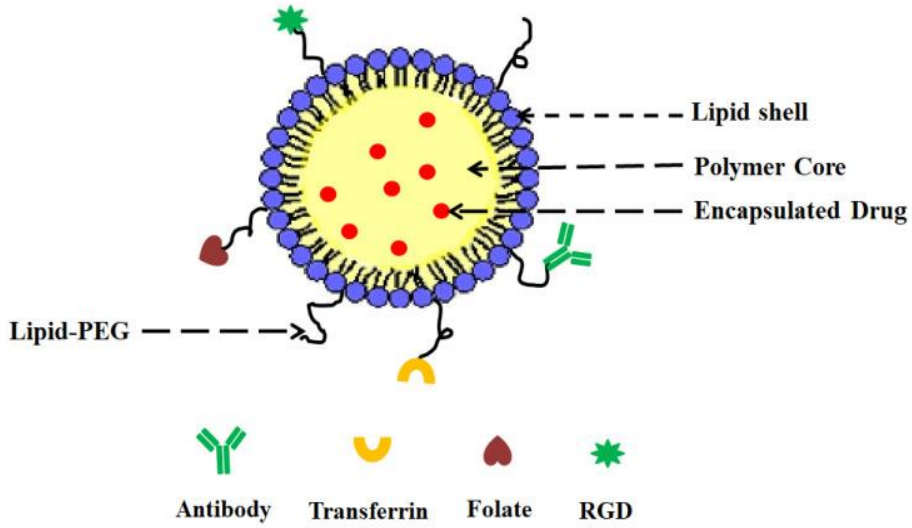
CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology enables precise genome editing by creating targeted double-strand breaks in DNA. The system utilizes the Cas9 nuclease guided by RNA to modify specific gene sequences.

The CRISPR-Cas9 system was adapted for genome editing by Jennifer Doudna and Emmanuelle Charpentier in 2012 (Jinek et al., 2012, p. 816–821).

Applications in cancer therapy include

Knockout of oncogenes
Correction of tumor suppressor mutations
Engineering immune cells (CAR-T therapy)

Although CRISPR offers permanent gene correction, challenges include off-target effects and safe delivery mechanisms.



1.2 RNA Interference (RNAi) Mechanism

Mechanism of siRNA-mediated gene silencing

Intracellular pathway

The first step of RNAi involves processing and cleavage of longer double-stranded RNA into siRNAs, generally bearing a 2 nucleotide overhang on the 3′ end of each strand. The enzyme responsible for this processing is an RNase III-like enzyme termed Dicer.[35-38] When formed, siRNAs are bound by a multiprotein component complex referred to as RISC (RNAinduced silencing complex).[39-42] Within the RISC complex, siRNA strands are separated and the strand with the more stable 5′-end is typically integrated to the active RISC complex. The antisense single-stranded siRNA component then guides and aligns the RISC complex on the target mRNA and through the action of catalytic RISC protein, a member of the argonaute family (Ago2), mRNA is cleaved.[43-47] (Figure 1).

Figure 1.

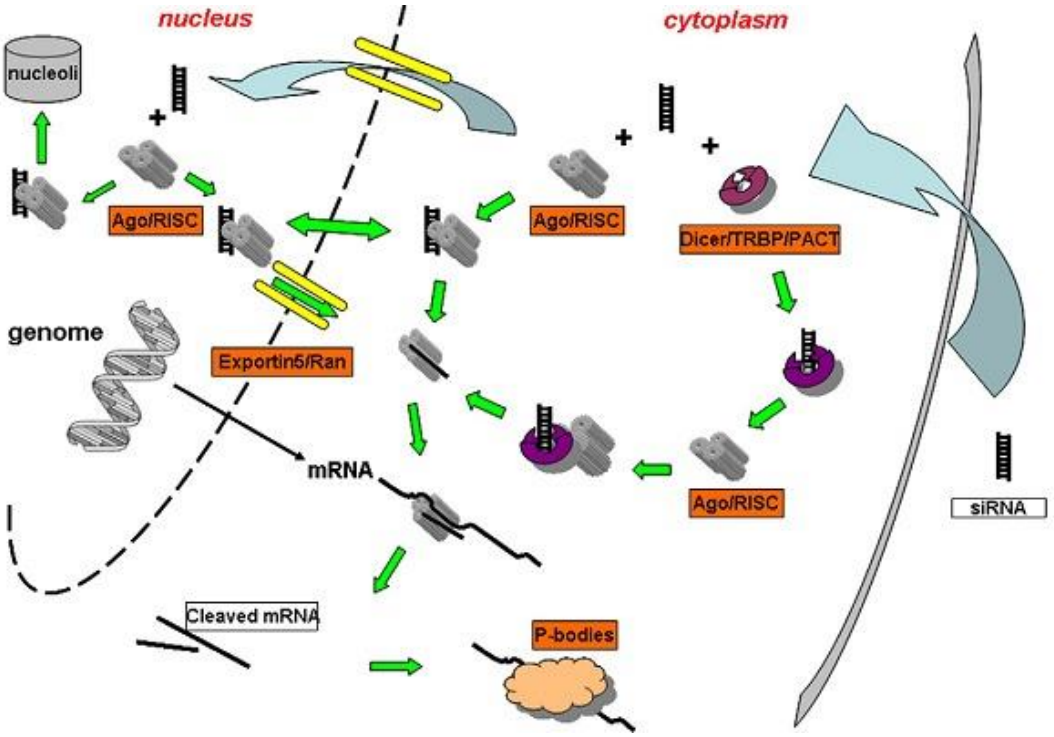


Figure 1: Schematic of the siRNA mediated RNA interference pathway.

Limitations of Conventional Gene Delivery

1. Viral Vectors: Immunogenicity & Mutagenesis

Viral gene delivery systems such as retrovirus, lentivirus, adenovirus, and AAV are effective at gene transfer but have important safety limitations. They can trigger strong immune responses, leading to inflammation and difficulty in repeated administration. Additionally, integrating viral vectors may insert their genetic material into the host genome **randomly**, causing **insertional mutagenesis** and potential oncogenesis by disrupting tumor suppressor genes or activating oncogenes. These issues, along with limited payload capacity and complex manufacturing, restrict their wider clinical application.

2. Instability of Naked siRNA

Unprotected siRNA is highly unstable in biological fluids. It is rapidly degraded by serum endonucleases and exonucleases, resulting in a **very short half-life in circulation** and poor therapeutic exposure. Chemical and formulation strategies are required to protect siRNA from degradation and enhance its stability for effective gene silencing.

3. Poor Cellular Uptake

siRNA and naked nucleic acids carry a negative charge and are hydrophilic, which prevents them from crossing the negatively charged cell membrane efficiently. Without a delivery vector, cellular entry is extremely limited, resulting in low intracellular delivery and reduced therapeutic effect. Encapsulation in nanoparticles or carriers significantly enhances uptake**.**

4. Rapid Degradation & Clearance

In addition to nuclease degradation, naked siRNA is rapidly cleared from systemic circulation through renal filtration, which significantly reduces **bioavailability** at target sites. This rapid elimination necessitates protective delivery systems to maintain therapeutic levels long enough to exert gene silencing.

Role of Nanotechnology in Gene Delivery

Nanotechnology has significantly improved the efficiency and safety of gene delivery systems, particularly for siRNA-based cancer therapy.

1. Protection from Nuclease Degradation

Nanocarriers such as lipid nanoparticles, polymeric nanoparticles, and lipid–polymer hybrid nanoparticles encapsulate siRNA within a protective matrix. This shielding prevents degradation by serum nucleases, increases circulation half-life, and enhances in vivo stability.

2. Enhanced Cellular Uptake

Nanoparticles improve intracellular delivery by facilitating endocytosis. Their optimized size (typically 50–200 nm) and surface charge promote interaction with the cell membrane. Surface modifications (e.g., PEGylation or ligand attachment) further enhance internalization efficiency.

3. Controlled Release

Nanocarriers can be engineered to release siRNA in a controlled or stimuli-responsive manner (e.g., pH-sensitive or enzyme-responsive release). This ensures

sustained gene silencing and minimizes premature drug leakage.

4. Targeted Delivery
Nanoparticles enable passive targeting via the Enhanced Permeability and Retention (EPR) effect in tumors, as well as active targeting through ligand conjugation (e.g., antibodies, peptides, folate). This improves accumulation in cancer tissues while reducing off-target effects and systemic toxicity.

Lipid Nanoparticles in Gene Therapy
Lipid nanoparticles (LNPs) are among the most clinically advanced non-viral gene delivery systems. They are widely used for delivering siRNA and mRNA due to their high encapsulation efficiency, biocompatibility, and ability to facilitate cytoplasmic release.

Mechanism of Endosomal Escape
After systemic administration, LNPs are internalized into cells primarily through endocytosis. Once inside endosomes, the **ionizable lipids** within LNPs become protonated in the acidic endosomal environment. This protonation promotes electrostatic interaction with negatively charged endosomal membrane lipids, leading to membrane destabilization and disruption. Consequently, siRNA is released into the cytoplasm, where it incorporates into the RNA-induced silencing complex (RISC) to mediate gene silencing. Efficient endosomal escape is critical, as endosomal entrapment is a major barrier in gene delivery.

Clinical Relevance (e.g., Onpattro)
The clinical success of lipid nanoparticle systems was demonstrated by Onpattro **(patisiran)**, the first FDA-approved siRNA therapeutic in 2018 for hereditary transthyretin-mediated amyloidosis. Onpattro utilizes an LNP formulation to protect siRNA from degradation and enable targeted delivery to hepatocytes in the liver. This milestone validated LNPs as a safe and effective platform for systemic gene therapy and accelerated the development of LNP-based RNA therapeutics.

1.6 Lipid–Polymer Hybrid Nanoparticles
Lipid-Polymer Hybrid Nanoparticles (LPHNPs) are advanced core-shell nanocarriers that combine the structural robustness of polymeric nanoparticles with the biocompatibility of lipid-based systems. They offer significant synergistic benefits for drug and gene delivery, notably enhanced mechanical stability and high transfection efficiency.

Structural Architecture
LPHNPs typically consist of three primary building blocks.

- **Prolonged Circulation:** The outer PEG layer allows the nanoparticles to evade the immune system (macrophage uptake) and circulate longer in the bloodstream.

Synergistic Benefits: Stability + Transfection Efficiency
The hybrid design overcomes the individual limitations of both liposomes (which often suffer from premature leakage and poor stability) and polymeric nanoparticles (which often exhibit low transfection efficiency).

- **Enhanced Structural Stability:** The polymeric core serves as a rigid cytoskeleton, providing mechanical stability and preventing drug leakage, which makes the nanoparticles highly stable during storage and systemic circulation.
- **Optimized Transfection Efficiency:** The lipid shell behaves like a natural cell membrane, greatly improving cellular uptake through endocytosis and facilitating endosomal escape. Meanwhile, the polymer core securely protects delicate genetic material (like mRNA, siRNA, or large plasmid DNA) from enzymatic degradation, ensuring the cargo reaches the cytoplasm intact.

1.7 Breast Cancer and Target Gene Selection
Gene silencing (via siRNA, shRNA, or ASOs) selectively blocks the expression of disease-driving proteins. In breast cancer therapy, it is critical for disabling hyperactive oncogenes, reversing chemoresistance, and inducing cancer cell death without damaging surrounding healthy tissues.

Importance of Gene Silencing in Cancer

- **Precision Targeting:** Unlike broad chemotherapies, gene silencing targets specific messenger RNAs (mRNAs), preventing the translation of mutated or overexpressed proteins responsible for tumor growth.
- **Induction of Apoptosis:** Cancer cells frequently develop mechanisms to evade programmed cell death. Silencing specific pro-survival genes helps to restore this natural cell death pathway.
- **Overcoming Chemoresistance:** Gene silencing can downregulate proteins that protect tumors, resensitizing drug-resistant breast cancer cells to standard treatments like paclitaxel or doxorubicin.
- **Inhibition of Metastasis:** By silencing genes that regulate cell migration and tissue invasion, therapies can restrict the ability of the tumor to spread.

Selection of Target Genes: BCL-2 and VEGF
1. BCL-2 (B-cell lymphoma 2)

- **Role in Cancer:** BCL-2 is a primary anti-apoptotic protein. In many breast cancers, its overexpression prevents cancer cells from dying, leading to prolonged tumor survival and therapy resistance.
- **Effect of Silencing:** Silencing BCL-2 using targeted siRNA downregulates its expression, causing mitochondria to release pro-apoptotic proteins and triggering cancer cell death. This mechanism suppresses tumor growth and enhances chemosensitivity.

2. VEGF (Vascular Endothelial Growth Factor)

- **Role in Cancer:** VEGF is the primary driver of angiogenesis (the formation of new blood vessels). Tumors overexpress VEGF to hijack the host's vascular system, ensuring a constant stream of oxygen and nutrients for unchecked growth.
- **Effect of Silencing:** Silencing the *VEGF* gene via RNA interference (RNAi) disrupts the secretion of the VEGF protein at the post-transcriptional level. This effectively "starves" the tumor, deprives it of survival signals, and halts angiogenesis

1.8 Research Gap

Developing safer, more efficient **non-viral delivery systems** is a critical priority in modern gene therapy and genome editing. While viral vectors are highly effective at introducing genetic material into cells, they carry significant risks—including robust immune responses, insertional mutagenesis (cancer risks), and limitations on repeated dosing.

Non-viral carriers—such as **lipid nanoparticles (LNPs)**, **cationic polymers**, and **exosomes**—offer superior safety profiles, lower immunogenicity, easier scalability, and flexible payload capacities. Despite these advantages, their widespread clinical translation is currently limited by the following research gaps and challenges.

Key Research Gaps

- **Low Transfection Efficiency:** Non-viral vectors generally exhibit lower transfection and transduction rates compared to viral vectors. They struggle to efficiently cross the cell membrane, escape the endosome, and traffic genetic cargo (like plasmid DNA or CRISPR machinery) to the nucleus.
- **Off-target Biodistribution:** Many non-viral systems accumulate primarily in the liver (hepatic clearance), making it difficult to achieve targeted, localized delivery to other vital organs, tumors, or the central nervous system (CNS) without employing complex tissue-specific modifications.
- **Systemic Instability & Clearance:** Positively charged non-viral complexes can aggregate in physiological fluids and are rapidly cleared from systemic circulation by the immune system (mononuclear phagocyte system).
- **Immunotoxicity & Cytotoxicity:** While inherently safer than viruses, some synthetic polymers (e.g., high molecular weight PEI) can induce cellular toxicity at effective doses, highlighting the need for highly biocompatible, biodegradable materials.

The Need & Current Directions

To bridge the gap between the *safety* of non-viral systems and the *efficiency* of viral vectors, ongoing research focuses on.

- Engineering **ionizable lipids** to enhance endosomal escape and cellular uptake.
- Developing **biomimetic nanocarriers** and **hybrid systems** (combining viral and non-viral properties) for precision targeting.
- Attaching targeting ligands and stimuli-responsive elements for controlled, site-specific release of therapeutics

Aim of the Study To develop and evaluate lipid–polymer hybrid nanoparticles for efficient siRNA delivery in breast cancer cells.

Lipid–polymer hybrid nanoparticles (LPHNPs) are engineered to overcome the instability and poor cellular uptake of siRNA. By combining a stable polymeric core with a biocompatible lipid shell, LPHNPs protect siRNA from degradation, enhance cellular endocytosis, and facilitate specific oncogene silencing in breast cancer cells.

Developing these nanoparticles typically follows a rational design framework to maximize therapeutic efficacy.

- **Structure:** A biodegradable polymeric core (such as PLGA or PLA) encapsulates the siRNA, surrounded by a lipid-PEG monolayer for prolonged circulation and immune evasion.
- **Targeting:** Surface functionalization with ligands or antibodies (like EGFR) enhances receptor-mediated endocytosis specifically into breast cancer cells.
- **Optimization:** Using software like Design Expert, researchers optimize the mole fractions of lipid components—such as DOPE and ionizable lipids—to improve nanoparticle stability and uptake.
- **Evaluation:** *In vitro* assessments (e.g., using MCF-7 cells) measure gene silencing efficacy, cytotoxicity, and cellular internalization to ensure targeted, off-target-free protein knockdown

Objectives of the Study To formulate siRNA-loaded lipid–polymer hybrid nanoparticles.

1. Formulation & Optimization

- **Fabrication Method:** Utilize single-step nanoprecipitation or a double-emulsion solvent evaporation technique to create a solid polymeric core enveloped by a lipid-PEG shell.
- **Optimization:** Employ **Design of Experiments (DoE)** methodologies (e.g., Box-Behnken design) to optimize key variables such as the lipid-to-polymer ratio, polymer molecular weight, and siRNA concentration to maximize delivery efficiency.

2. Physicochemical Characterization

- **Size, PDI, and Zeta Potential:** Determine particle size, Polydispersity Index (PDI), and surface charge using **Dynamic Light Scattering (DLS)**.
- **Morphology:** Visualize the core-shell architecture, shape, and surface smoothness using **Transmission Electron Microscopy (TEM)**.

3. Encapsulation Efficiency (EE)

- **Quantification:** Separate unencapsulated siRNA from the nanoparticles using ultrafiltration or size-exclusion chromatography. Determine the encapsulation efficiency using a fluorescence-based assay (e.g., **RiboGreen**) or UV-spectroscopy.

4. In Vitro Release Studies

- **Profile Generation:** Perform *in vitro* release assays using the **dialysis bag method** against a physiological buffer.

5. Cellular Uptake

- **Visualization and Quantification:** Transfect target cells with fluorescently labeled siRNA-loaded nanoparticles. Assess intracellular internalization and localization using **Confocal Laser Scanning Microscopy (CLSM)** and **Flow Cytometry**.

6. Gene Silencing Efficiency

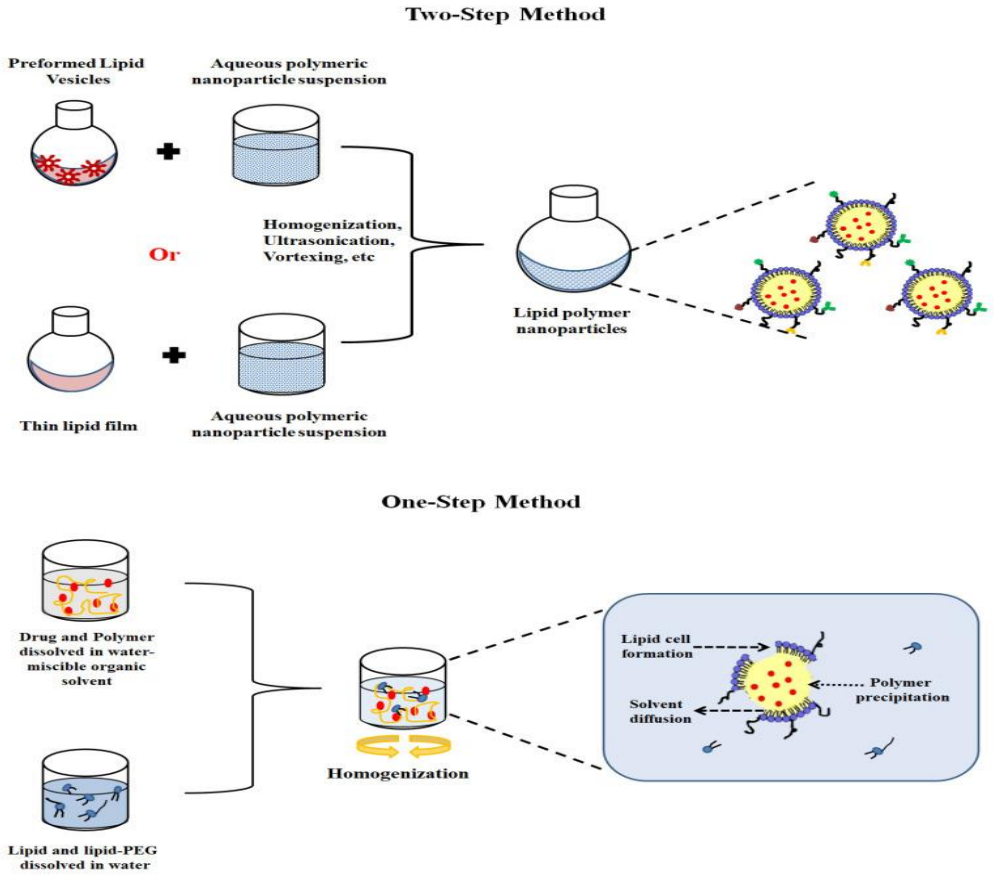
- **mRNA and Protein Assessment:** Evaluate the efficacy of target gene knockdown in the chosen cell line. Use **RT-qPCR** to measure mRNA degradation levels and **Western Blotting** for target protein expression.

7. Cytotoxicity and Apoptosis

- **Cell Viability:** Assess nanoparticle biocompatibility across varying concentrations using standard metabolic assays (e.g., **MTT or WST-1**).
- **Apoptosis:** Quantify apoptotic cell death using **Annexin V/Propidium Iodide (PI)** staining flow cytometry to confirm nanocarrier safety.

LPHNP Fabrication Methods

LPHNPs have been prepared traditionally by a simple two-step method, but nowadays, a transformation strategy has been introduced that transitions a two-step LPHNPs preparation to a one-step strategy. The latter technique synchronously uses the self-assembly of polymers and lipids. Owing to their two-in-one structure, these LPHNPs are used for combinatorial drug delivery, especially in oncology. The outer lipid–PEG layer can be tailored in such a fashion to achieve the targeting of anticancer therapy, delivery of nucleic acids, and to be used in diagnostic imaging. Although the fusion mechanism of lipid and polymer is still unclear, methods of LPHNP preparation that utilize different mechanisms of formation are presentedin Figure



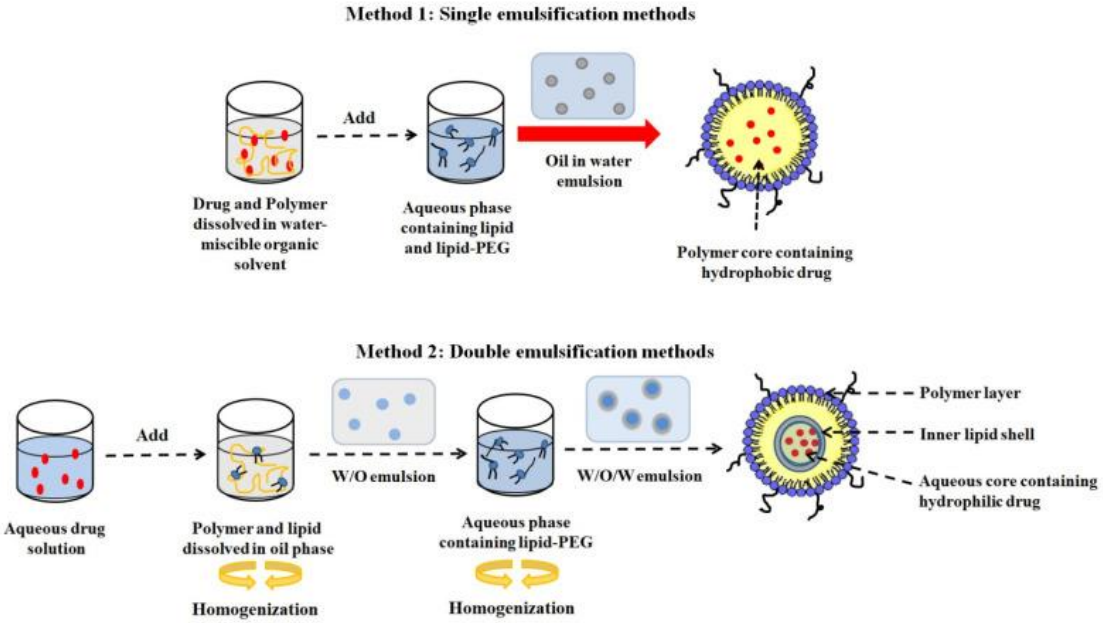
In the two-step method, the LPHNPs are produced by either directly adding the aqueous polymeric nanoparticle suspension to the dried lipid film or first hydrating the thin lipid film with an aqueous solvent to produce lipid vesicles, and then these lipid vesicles are added to an aqueous preformed nanoparticle suspension.

In both methods, the hybrids are assembled by the input of external energy provided via vortexing or through the ultrasonication of the suspension and heating at a temperature beyond the phase transition temperature of the lipid constituent. The prepared hybrid structure is thermodynamically stable via electrostatic, hydrophobic, and van der Waals interactions.

In the single-step method, the drug and polymer solution in a water-miscible organic solvent is added to an aqueous medium encompassing lipids or lipid–PEG, with spontaneous homogenization, resulting in self-assembly into a monolayer of lipids surrounding the core. During this stage, PEGylated lipids are also self-assembled, with a lipid moiety clinging to the surface of the polymer core and the PEG chain extending externally toward the aqueous environment.

The two-step method has the limitation of formulating PNPs and lipid vesicles separately, which requires more energy and is a more time consuming process. The single-step or one-step method is more prevalent and efficient as lipid vesicles and PNPs are not prerequisites in this method. In this method, the spontaneous self-assembled monolayer formation occurs, surrounding the core to form LPHNPs; therefore, it is a rapid and energy-efficient technique.

The emulsification solvent evaporation (ESE) method is another technique for the preparation of LPHNPs. This method is further classified into single and double emulsification methods. In the single ESE method, hydrophobic drugs and polymers dissolved in the oil phase are mixed with an aqueous phase containing lipids under constant agitation to form an oil-in-water (o/w) emulsion. The evaporation of the organic medium simultaneously forms a polymer core surrounded by a lipid layer. As an apparent substitute, the lipid can be dissolved in the oil phase alongside the polymer [52]. For water-soluble formulations, the double ESE method is employed to prepare w/o/w emulsion. In this method, the aqueous drug solution is emulsified in an organic solvent that contains polymers and lipids to form a w/o mixture. The prepared w/o mixture was further emulsified in a lipid–PEG-containing aqueous phase to form a w/o/w emulsion, followed by subsequent oil phase evaporation to produce LPHNPs. The LPHNPs produced by the double ESE method have certain structural anomalies, including: (1) aqueous core surrounded by a lipid layer; (2) a polymer layer in between; and (3) an outer lipid–PEG shell.



On the basis of the desired biomedical applications of LPHNPs, a lipid-based surface engineering method is to be chosen, which further depends on the nature of lipid–polymer surface chemistry. For example, the single-step ESE method is used for gene delivery applications, while the conventional two-step top-down approach is required for nanoghost-based surface engineering.

Nanoprecipitation technique is another method for the preparation of LPHNPs. This technique utilizes the dispersion of lipids in the aqueous phase followed by the addition of a drug–polymer solution in a water-miscible organic solvent with sonication, stirring, or homogenization. This causes the polymer to precipitate via solvent diffusion, as well as the lipid to self-assemble onto the polymeric nanoparticle interface. The nanoprecipitation approach was used to prepared triptolide (TL)- and paclitaxel (PTX)-loaded LPHNPs for lung cancer treatment. Prepared LPHNPs were approximately 160 nm in size with a 0.2 polydispersity index. Both drugs showed a greater encapsulating efficiency of about 85%, and the in vivo results revealed

around a four-fold reduction in tumor volume as compared with the control group (i.e., PTX and TL). The findings indicate synergy as well as a greater efficiency in treating lung cancer. To achieve a homogenous liquid crystalline phase, the temperature must be kept above the gel-to-sol temperature of lipids during the synthesis. The lipid–aqueous phase acts as an anti-solvent (above their transition temperature) toward a dropwise injection of polymer in organic solvent, resulting in the polymer to aggregate and coil, followed by a self-assembly of lipids surrounding the polymeric core.

Lipid/polymer ratio plays an imperative role in the synthesis of LPHNPs. As the phospholipid concentration rises over the critical micelle concentration, liposomes form, while decreasing the L/P ratio causes agglomeration due to the anti-solvent impact of the aqueous phase, which reduces lipid content for interfacial stabilization. Furthermore, the use of PEGylated lipids increases the colloidal stability of hybrid NPs. Because of the steric stabilization provided by PEG chains, the PEGylated lipid increases stability without interfering with drug loading and release.

Numerous LPHNP studies are listed in describing the preparation methods of LPHNPs, encapsulating materials, targeting ligands, physicochemical properties, and their applications.

CONCLUSION

Lipid–polymer hybrid nanoparticles (LPHNs) represent a highly promising platform for the targeted delivery of small interfering RNA (siRNA) in cancer therapy. By integrating the structural stability and controlled-release properties of polymeric nanoparticles with the excellent biocompatibility and membrane fusion capabilities of lipid-based carriers, LPHNs effectively overcome many of the biological barriers that limit the clinical application of siRNA. These hybrid systems enhance siRNA protection from enzymatic degradation, improve cellular uptake, facilitate endosomal escape, and enable selective accumulation within tumor tissues through passive and active targeting strategies. Recent advances in surface functionalization, stimulus-responsive formulations, and co-delivery approaches have further improved their therapeutic efficacy while minimizing systemic toxicity.

Despite these encouraging developments, several challenges remain, including large-scale manufacturing, long-term safety evaluation, formulation reproducibility, regulatory approval, and clinical translation. Future research should focus on optimizing nanoparticle design, improving tumor-specific targeting, and validating therapeutic outcomes through well-designed clinical studies. Overall, lipid–polymer hybrid nanoparticles have the potential to revolutionize precision oncology by providing a safe, efficient, and personalized platform for siRNA-based cancer treatment, paving the way for next-generation gene-silencing therapeutics.

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