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A REVIEW OF LIPID-POLYMER HYBRID NANOPARTICLES AS A NEW GENERATION DRUG DELIVERY SYSTEM

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ABSTRACT: Lipid–polymer hybrid nanoparticles (LPNs) are next-generation core-shell nanoparticle structures comprising polymer cores and lipid/lipid–PEG shells, which produce complementary characteristics of both polymeric nanoparticles and liposomes, specifically in terms of their physical stability and biocompatibility. Recently the LPNs have been shown to have superior in vivo cellular delivery efficacy compared to that obtained from polymeric nanoparticles and liposomes. Since their inception, the LPNs have advanced significantly in terms of their preparation strategy and scope of applications. LPN preparation has undergone a significant shift from the traditional two-step method to the now widely used two-step method, which is more complex principal-wise and yet easy to perform, relying on self-assembly of lipid and polymer. This has resulted in a better product and high production throughput as well. Unlike the initial days when LPNs were used mainly for single drug delivery in anticancer therapy, now they are widely used in combinatorial and active drug targeted delivery, delivery of genetic material, vaccines, and in advanced diagnostic procedures as well. This review throws light on the most recent state of development of the preparation and application of LPNs and the future works to be done to make the LPNs more affordable and reliable for their widespread use and to use their full potential for the betterment of mankind.

INTRODUCTION: Nanotechnology can be defined as the study and use of structures between 1 nanometer and 100 nanometers in size. It represents a relatively new and powerful tool in the field of medicine to treat a large variety of diseases, such as cardiovascular, cancer, and CNS diseases. Thus, it is not surprising that nanoscale particles (<1000nm) have been used extensively as drug delivery vehicles for various therapeutic substances, such as diagnostic imaging agents, small molecule drugs, genes and biopharmaceuticals, *etc.* [1].

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Small nanoparticles can evade capturing by the reticuloendothelial system (RES) resulting in higher bioavailability [2]. Furthermore, nano nano-particles are very helpful in crossing blood-brain barrier also.

The reason behind the widespread use of nanoparticles is primarily due to their small size that results in high therapeutic efficacy in delivering drugs to their desired site of action [3]. Polymers (*e.g.*, Polymeric nanoparticles, polymeric micelles, dendrimers) [4-6], lipids (*e.g.*, Liposomes, solid nanoparticles) [7-9], and metals (*e.g.*, gold, silica) [10, 11] are commonly used as nanocarriers. Among the nanocarriers, the most prominent are the polymeric nanoparticles and liposomes, attributed to their advantageous characteristics as discussed below.

Polymeric nanoparticles are commonly used as they have some properties like high structural integrity, stability during storage, and controlled release capability. Polymeric nanoparticles are also easy to prepare and can be used in active targeted delivery easily [12]. Polymeric nanoparticles can be prepared from natural polymers (*e.g.*, Chitosan) and synthetic biodegradable and biocompatible polymers (*e.g.*, poly-lactic-co-glycolic acid (PLGA)).

Compared to polymeric nanoparticles liposomes are considered as the more fanciful drug delivery vehicles because of their excellent biocompatibility as liposomes are basically analogues of biological membranes. Liposomes can be prepared from both natural and synthetic phospholipids [13]. But the liposomes are easily cleared by the RES, resulting in poor bioavailability [14]. Hence lipid-PEG is often used in liposome preparation to enhance *in-vivo* circulation [15, 16]. But liposomes show content leakage and instability during storage because of lack of structural integrity [17].

A new generation of therapeutic drug delivery, namely Lipid-polymer hybride nanoparticles (LPNs) has been developed to overcome the difficulties and limitations related to the nanoparticles and liposomes [18]. LPNs have combined characteristics of both lipid polymers and loposomes.

They are made of three components as

i. A polymer core in which the therapeutic substances are encapsulated,

ii. An inner lipid layer enveloping the polymer core, the main function of which is to provide biocompatibility to the polymer core, and

iii. An outer lipid–PEG layer, which functions as a stealth coating that enhances *in-vivo* circulation time of the LPNs, as well as providing steric stabilization. Moreover, In addition, the inner lipid layer also functions as a molecular barrier that minimizes leakage of the encapsulated content during the LPNs preparation. Furthermore, the inner lipid layer slows down the polymer degradation rate of the LPNs product by limiting inward water diffusion, thus enabling the sustained release of the content.

Because of having this core-shell structure, the LPNs show (i) high structural integrity, stability during storage, and controlled release properties due to the polymer core and (ii) high biocompatibility and bioavailability because of the lipid and lipid–PEG layers [19]. Because of these characteristics, the LPNs have become a very popular and reliable means of drug delivery.

In this review, we discuss the preparation methods of LPNs, which can broadly be classified into two basic categories one step and two-step methods and various applications of LPNs. Moreover, the use of LPNs as delivery vehicles of genetic materials and diagnostic imaging agents are also included in this review.

2. Preparation of LPNs:

2.1. Two-Step Method:

2.1.1. Conventional Two-Step Method: It was the most common method used in the early phase of LPNs development. In the conventional two-step method, preformed lipid vesicles are adsorbed on the preformed polymeric nanoparticles by electrostatic interactions.

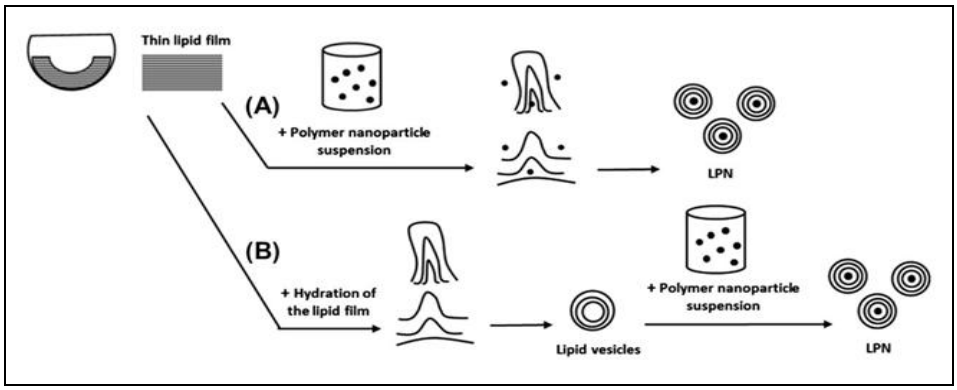

FIG. 1: TWO ROUTES OF LPNS PREPARATION BY THE TWO-STEP METHOD – (A) ADDING AQUEOUS POLYMERIC NANOPARTICLE SUSPENSION TO THIN LIPID FILM; (B) MIXING THE AQUEOUS POLYMERIC

The polymeric nanoparticles are typically prepared either by nanoprecipitation [20], emulsification-solvent-evaporation [21] or high-pressure homogenization [22]. The LPNs in the two-step method can be prepared by adding the previously formed polymeric nanoparticles to (A) a dried thin lipid film, where the lipid film is prepared by dissolving the lipid in an organic solvent (*e.g.*, chloroform), followed by evaporation in a rotary evaporator [20], or alternatively added to (B) preformed lipid vesicles prepared by hydration of the thin lipid film.

In both the methods LPNs are prepared at a temperature higher than the gel-to-liquid transition temperature lipid by either vortexing or ultrasonication of the mixed polymer-lipid suspension. Then by centrifugation LPNs are separated from non adsorbed lipid. To obtain uniformly sized LPNs, The LPNs suspension after preparation are often subjected to homogenization or extrusion steps. In the extrusion method, the LPNs suspension is passed through a porous membrane to produce LPNs in the range of size of the membrane's pores [23].

2.1.2. Non-Conventional Two-Step Method: In non-conventional methods like (ii) spray drying and (i) soft lithography particle molding and ii) spray drying have been used to prepare LPNs in a non-convention way. Particle Replication in Non-Wetting Templates (PRINT), a soft lithography particle molding technique, was used to prepare LPNs for gene delivery [24]. This technique uses a organic solvent (*e.g.*, dimethyl sulfoxide (DMSO) or dimethylformamide (DMF)) to dissolve the polymer (*i.e.*, PLGA) with the genetic material siRNA and cast it onto a polyethylene terephthalate (PET) sheet. Then keeping the PET sheet in contact with a PRINT molding with 80-320 nm patterns and heated. The PLGA flows into the mold and solidifies when kept in ambient temperature, and forms PLGA-nanoparticle. These are then harvested by keeping the PET sheet in contact with a PVA coated PET sheet. Then aqueous solution of the lipids (*i.e.*, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)) was used to dissolve the PVA layer and release the nanoparticles from the mold and simultaneously forming lipid-coated PLGA nanoparticles. The PRINT technique enables precise control over size and shape of the LPNs as these properties are governed by the size and shape of the mold cavity. It is also capable of producing mono-disperse nanoparticles of different aspect ratios.

Hitzman *et al*., [25] mentions preparation of polymeric nanoparticles (*i.e.*, polyglutamic acid, polylysine) (400-500 nm) by spray drying after which they were dispersed in dichloromethane solution containing the lipids (*i.e.*, tripalmitin, tristearin, cetyl alcohol). The lipid–polymer suspension was then spray-dried to produce lipid-coated polymeric nanoparticles.

2.2. One-Step Method: In terms of energy and time used, the two-step method used for the preparation of nanoparticles is not efficient as they need readily available and separately prepared preformed polymeric nanoparticles and lipid polymers and thus, it necessitated the development of a new method known as One-step method.

It does not need preformed Polymeric nanoparticles and lipid vesicles. Instead, the one-step method only needs mixing of polymers and lipid solution,, after which they self assemble to form LPNs by either nanoprecipitation or emulsification solvent evaporation.

2.2.1. One-Step Method by Nanoprecipitation: In one-step method, the polymer and the to-be-encapsulated substance are dissolved in a water-miscible organic solvent (*e.g.*, acetone), while the lipid and/or the lipid–PEG are dispersed in water in conventional nanoprecipitation technique. A homogenous solution of lipid and lipid–PEG is prepared by heating to 65-70 °C. And then, the polymer solution is added dropwise to the aqueous lipid dispersion under continuous stirring, which causes the polymer to precipitate into nanoparticles.

Simultaneously, the lipids self-assemble around the polymeric nanoparticles because of hydrophobic interactions. The hydrophobic tail of the lipids is attached to the polymer core, and the hydrophilic head sticks out to the external aqueous surrounding. This results in the formation of lipid-stabilized LPNs [26]. After the evaporation of the solvent, the resultant LPNs are recovered by centrifugation.

2.2.2. Recent Advances in Nanoprecipitation: Fang *et al*., [27] developed a rapid nanoprecipitation process which increased the productivity by twenty folds compared to conventional methods by supplying a high and uniform level of energy input via bath sonication. Their method uses a small amount of organic solvent, which evaporates during the assembly process itself, resulting in a much lesser time to recover the LPNs than in the conventional methods.

Valencia *et al*., [28] carried out the nanoprecipitation in a microchannel (300 µm height) to improve the size homogeneity of LPNs. This microscale mixing resulted in highly uniform LPNs in size, and it provided uniform lipid coverage of the lipid core. Kim *et al*., [29] did a follow-up study that addressed the main drawback of this procedure, *i.e.*, low throughput. They used a large channel of height 2000µm and did nanoprecipitation at Reynolds number (Re) 75. In addition, the control over the LPNs size was enhanced by regulating the micro vortex formation by manipulating the 3D fluid flow patterns inside the microchannel.

Though recent advances in the nanoprecipitation method have made it highly effective and capable of large-scale production, yet still nanoprecipitation method remains limited to applications in which the substance to be encapsulated is soluble in water-miscible organic solvents (*e.g.*, acetone, acetonitrile) [30]. Moreover, encapsulation of water-soluble substances by nanoprecipitation led to the leakage of the aqueous phase and thus poor encapsulation efficacy [30]. So, an alternative one-step LPNs preparation method by emulsification–solvent–evaporation (ESE) was developed, which enables the encapsulation of a wide range of substances.

2.2.3. One-Step Method by Emulsification-Solvent-Evaporation (ESE):

ESE can be Classified into two Types (A) Single ESE and (B) Double ESE: A single ESE technique is used when the substance to be encapsulated soluble in a water-immiscible solvent (i.e., oil phase). In this method, under constant stirring or ultrasonication, the oil phase, which contains the polymer and the substance to be encapsulated, is added into an aqueous phase containing the lipid to form an oil-in-water (o/w) emulsion. LPN is produced at last after oil in solution gets evaporated and lipid simultaneously self assembles around the polymer core [31]. Alternatively, the lipid can also be dissolved in the oil phase together with the polymer [30].

A double ESE method is used when the substance to be encapsulated is insoluble in any organic solvents, and thus it cannot be dissolved together with the polymer. For these kinds of substances, it is dissolved in the aqueous phase and emulsified in an oil phase containing the polymer and the lipid. This w/o emulsion is emulsified again in an aqueous phase containing the lipid–PEG to form w/o/w emulsion. After evaporation, this produces LPNs. The LPNs produced by the double ESE method has a slightly different structure. They are comprised of (a) an inner lipid layer around the aqueous hollow core, (2) a middle polymer layer, and (3) an outer lipid–PEG layer.

3. Applications of Lipid Polymer hybrid Nanoparticles:

3.1. Drug Delivery: The different preparation methods available for LPNs have enabled the encapsulation of a wide range of drugs, regardless of their aqueous solubility, ionicity, hydrophilicity, and lipophilicity as demonstrated in Cheow *et al*., [30]. In this section, the drug delivery applications of LPNs are classified into three subsections, which are (1) single, (2) combinatorial drug deliveries, and (3) active targeted drug delivery by LPNs.

3.1.1. Single Drug Delivery: Many studies have employed LPNs to deliver single chemo-therapeutic drugs tested against different types of cancer cells (*e.g.*, breast, prostate, lung, liver, cervical) [19, 25, 26, 32, 33]. The focus of a majority of the single drug delivery studies were on *in-vitro* cytotoxicity of drug-loaded LPNs [32], the biocompatibility of drug-free LPNs [19], cellular uptakes [26, 33], and drug release kinetics [25]. For example, Chih-Hang *et al*., [33] and Zhang *et al*., [26] respectively, reported that the LPNs exhibited higher uptakes in cervical and prostate cancer cells than the non-hybrid polymeric nanoparticles. Liu *et al*., [32] prepared LPNs encapsulating anticancer drug – PCX – showing sustained release kinetics (*i.e.*, 33% in 12 h, 100% in 7 days). Due to longer drug exposure of the cancer cells compared to free drug, this sustained-release formulation showed higher

cytotoxicity (6-7 fold higher) against MCF7 breast cancer cells after 24 and 72 h incubation. LPNs showed 70% higher cellular uptake than non-hybrid PLGA nanoparticles after 4 h of incubation. LPNs also showed more effective drug release compared to non-hybrid preparation. The fact that liposomoes can penetrate the thick mucus layers surrounding bacterial colonies motivated the development of LPNs formulation to deliver fluoroquinolone antibiotics for the treatment of lung infections for higher local antibiotic exposure.

3.1.2. Combinatorial Drug Delivery: Development of a single nanoparticle carrier system that can carry multiple agents at precise composition ratios with controlled-release capability while remaining bio-compatible, such as LPNs, has helped to overcome the limitations of delivering a combination of different drugs or different chemotherapeutic agents with drugs.

Wang *et al*., [34] developed LPNs (440 nm) using the co-delivery of DOX and DNA. In their approach, DOX was encapsulated in the PLGA nanoparticles and the DNA was electrostatically bound to the cationic lipid shell of the LPNs (*i.e.,* PEG-OQLCS/FA-OQLCS/Chol) post-LPNs preparation. The LPNs showed slow release of DOX and were more effective in killing MDA-MB-31 breast cancer cells *in-vitro*. They also showed more cellular uptake and higher transfection efficiency.

Sengupta *et al*., [35] developed LPNs for a combinatorial delivery of anti-angiogenesis {cobretastatin (COM)} and chemotherapeutic drug (DOX). If the two drugs were delivered separately, the chemotherapeutic drug could not reach the tumor cells once the anti-angiogenesis action took place due to the cut-off blood vessels. Thus, it was crucial that the tumor cells were exposed simultaneously to the two drugs *via* the combinatorial delivery approach. Using a two-step method, they covalently conjugated COX with PLGA (polymer), and COM was inserted in preformed lipid vesicles (*i.e.,* PC/Chol/DSPE-PEG). In vivo results showed that mice incubated with B16/F10 melanoma or Lewis lung carcinoma cells exhibited greater tumor growth inhibition and longer lifespan when they were treated with the LPNs, compared to treatments with DOX and COM-loaded liposomes, which was due to the simultaneous burst release of DOX together with COM in liposomes.

Same approach Aryal *et al*., [36] covalently linked DOX and CMT to PLA chains of the same length prior to the nanoprecipitation steps. They found that the LPNs (100 nm) exhibited higher *in-vitro* cytotoxicity against MDA-MB-435 breast cancer cells compared to the free drug-polymer conjugates (*i.e.,* not transformed into LPNs). This preparation could escape the cells' efflux pump because of their uptake by endocytosis. As a result, higher cytotoxicity was seen.

Wang *et al*., [37] developed LPNs (65 nm) namely ChemoRad for the co-delivery of chemotherapeutic drug DCX and radiotherapy agents (*i.e.,* Indium-111 or Yttrium-90) using nanoprecipitation method. The preparation was easily taken up by LNCaP prostate cancer cells within 45 min. ChemoRad LPNs exhibited higher in vitro cytotoxicity than the LPNs containing only one of the agents.

A variety of drug combinations have been successfully conjugated using hydrolyzable linkers and subsequently incorporated into LPNs. For example, LPNs encapsulating hydrolyzable PCX-gemcitabine HCl [38] and PCX-cisplatin conjugates [39] have been prepared. On this note, the endocytic uptake mechanism of the LPNs was particularly favorable for drugs conjugated by hydrolyzable linkers. On endosomal maturation into lysosomes, pH drops, and it exposes the drug conjugates to an acidic environment that promotes the cleavage of the hydrolyzable linkers and release of the individual drugs.

Aryal *et al*., [39] found that the LPNs (70 nm) loaded with the PCX-gemcitabine HCl conjugates exhibited a higher level of *in-vitro* cytotoxicity against XPA3 pancreatic cancer cells than the free drug conjugates.

Aryal *et al*., [39] also reported that the LPNs (70 nm) loaded with the PCX-cisplatin conjugates showed higher *in-vitro* killing efficacy (60% killed) against A2780 ovarian cancer cells than the free drug conjugates (5% killed). The higher cytotoxicity of these LPNs was again attributed to their ability to bypass the clearance by the cell's efflux pump.

3.1.3. Active Targeted Drug Delivery: Active targeted drug delivery, sometimes called smart drug delivery aimed at maximizing drug exposure to the desired organ or target cells whilst minimizing the potential adverse effects on the healthy tissue. In the case of LPNs delivery platform, they are tagged with targeting molecule(s) such as folic acid. The PEG end group of the lipid–PEG layer of the LPNs is suitable for conjugation of the targeting moieties [40]. The targeting moieties can be conjugated to the lipid or lipid–PEG precursors (*i.e.*, pre-insertion technique) prior to the LPNs preparation or conjugated to the lipid shell of the LPNs post-LPNs preparation (*i.e.*, post-insertion technique).

Messerschmidt *et al*., [23] conjugated a chemo-therapeutic drug (*i.e.*, single-chain tumor necrosis factor (scTNF)), to the polystyrene nanoparticle core of the LPNs. They used single-chain variable fragments (scVF) as the targeting moiety and this produced a four fold increase *in-vitro* cytotoxicity owing to increased uptake by FAP expressing HT1080 fibrosarcoma cells.

Liu *et al*., [41] conjugated FA to the lipid–PEG precursor to prepare FA-conjugated LPNs encapsulating DCX, which was made up of PLGA core and DLPC/DSPE–PEG lipid shell. The FA inclusion resulted in slightly larger LPNs (260 nm) but the zeta potential, drug encapsulation efficiency, and release kinetics were minimally affected.

Due to the FA receptor-mediated endocytosis, the FA-conjugated LPNs exhibited 54% higher cellular uptake in MCF7 breast cancer cells after 2 h incubation compared to the bare LPNs in turn showing 51% higher in vitro cytotoxicity. Similarly, Zhao *et al*., [21] conjugated FA with PCX loaded LPNs and reported enhanced cellular uptake in HeLa cervical cancer cells.

Apart from FA, other active targeting moieties, such as aptamers [18], single-chain variable fragment [23], antibody [42], transferring [43], and peptides [44], [45] have been conjugated to LPNs and successfully demonstrated active targeted delivery. For example, Zhang *et al*., [18] used A10 RNA aptamer to target prostate-specific membrane antigen (PSMA) overexpressed in some prostate cancer cells.

Hu *et al*., [42] conjugated anti-carcinoembryonic (anti-CEA) half-antibody to PCX-loaded LPNs to target pancreatic cancer cells over expressing CEA using post-insertion technique resulting in two fold increase in in-vitro cytotoxicity. These LPNs selectively targeted CEA positive BxPC-3 pancreatic cancer cells. They used preformed LPNs made up of PLGA core and lecithin/DSPE–PEG lipid shell and conjugated them with anti-CEA half antibody. Similarly, Zheng *et al*., [43] conjugated transferrin (Tf) to pre-formed LPNs encapsulating aromatase inhibitor (7a-APTADD). This resulted in enhanced uptake by SKBR-3 breast cancer cells and hence lower IC_{50}.

Chan *et al*., [45] developed conjugated LPNs for the treatment of injured vasculature using the post-insertion technique. They used peptides as targeting moieties, which targeted the Collagen IV of the basement membrane. Chan *et al*., [44] demonstrated that these LPNs were effective in preventing recurrence of arterial restenosis.

3.2. Gene Delivery: Non-viral vehicles such as cationic lipids (*e.g.*, DOTAP) and cationic polymer (*e.g.*, PEI) are preferred for gene delivery as Non-viral vehicles over viral routes as the former has properties like low toxicity, low immunogenicity and are potential for large- scale production [46]. These non-viral vehicles are highly prone to non-specific binding with serum proteins resulting in poor delivery to the target organ due to their highly cationic surface. Hence, alternative non-viral formulations are needed with longer systemic circulation property post-administration. For this purpose, different techniques are being tried by coating them with polymer [47], using lipid-PEG instead of regular lipids, which reduces surface charge and encapsulation inside liposomes.

One such approach for lipoplexes is by coating the lipoplexes with a polymer to shield the lipoplexes from the non-specific protein binding [47]. Another approach is by using lipid–PEG in place of regular lipids, where the PEG aids in reducing the surface charge of the lipid/DNA complex. Using a similar concept, for the polyplexes, encapsulation of the polyplexes inside liposomes has been explored [48]. All these approaches have been shown effective *in-vivo* in reducing the non-specific protein binding of the DNA complexes.

Currently, LPNs studies for gene delivery are in the phase of identifying the best method of preparation and formulation.

3.2.1. DNA Delivery: Zhong *et al*., [49] evaluated three different DNA incorporation methods, in terms of the resultant transfection efficiency, in the preparation of LPN/DNA complex encoding luciferase gene (pLuc) namely (i) the 'OUT' method:- electrostatic adsorption of the DNA onto the cationic lipid shell of the LPNs (100–400 nm) post-LPNs preparation, (ii) the 'IN' method :- encapsulation of the DNA in the aqueous hollow core of the LPNs, and (iii) the 'BOTH' method :- a combination of the ''out'' and ''in'' methods. A total of six LPN/DNA complex formulations (i.e., three DNA incorporation methods each for the DOTAP and DC-Chol formulation) were evaluated.

Comparing the six LPN/DNA complex formulations, Zhong *et al*., [49] concluded that the 'OUT' method was ideal when an initial strong gene delivery response over a short period was required (*e.g.*, priming vaccination) and the 'BOTH' method was suitable for a situation in which a sustained response was needed (*e.g.*, booster vaccination).

Using the 'OUT' method, Li *et al*., [50] mixed preformed positively charged LPNs made up of PEI core and lipid shell (130-240 nm) with plasmid DNA encoding green fluorescent protein (pEGFP-N2) to prepare LPN-DNA complex. The LPN/DNA complex was successfully transfected into the HEK 293 cells, as well as MDA-MB-231 breast cancer cells. Significantly, the transfection efficiency of the LPN/ DNA complex was higher than that achieved by Lipofectamine. The complex was colloidally stable and exhibited minimal cytotoxicity toward the HEK 293 cells. They also reported that the PEGylated complex exhibited higher transfection efficiency than the non-PEGylated one (30–37% versus 18–19% transfected cells, respectively).

3.2.2. siRNA Delivery: siRNA has the ability to suppress the expression of specific genes by RNA interference and this is property is being used with great potential in anti-cancer therapies. For example, delivering siRNA to cancer cells can initiate the RNA interference pathway to inhibit the expression of proteins involved in tumor initiation and progression [51]. Numerous formulations have been developed for siRNA delivery following the same formulation path for DNA delivery (**i.e.,** lipoplexes, polyplexes) [52, 53]. However, they face similar challenges as those faced in DNA delivery (i.e., poor stability upon systemic administration). Thus, better siRNA delivery formulations, including LPNs, have continuously been explored.

Using the mentioned 'OUT' method like Zhong *et al*., [49], Yang *et al*., [54] used preformed LPN and siRNA to produce LPN-siRNA complex bound by electrostatic interaction between them. Two LPNs distinct in their polymer core formulations (*i.e.*, mPEG–PLA versus mPEG–PLA/PLA), where both were enveloped by BHEM-Chol lipid, were evaluated. The LPN/siRNA complex with the mPEG–PLA/PLA core exhibited better silencing of the Plk1 protein, which is often overexpressed in cancer cells, than LPN having mPED-PLA core. The *in-vivo* results in a BT 474 xeno-graft murine model confirmed the result.

Shi *et al*., [55] using a similar method like the 'IN' method used by Zhong *et al*., [49] encapsulated the siRNA inside the LPNs (230 nm) prepared using the double ESE method. LPNs made up of PLGA and egg PC/Lecithin/DSPE–PEG, showed ten times higher encapsulation efficiency of the siRNA at 80% (w/w) than the non-hybrid PLGA nano-particles. Despite the high siRNA loading (380 pmol PLGA/mg of PLGA), the LPNs exhibited sustained siRNA release, where 50% of the siRNA was released slowly over 12-20 h, because of having lipid bilayer. Subsequent *in-vivo* followed by *in-vitro* studies using different cell lines showed confirmatory results.

Hasan *et al*., [24] proposed using the aforementioned two-step PRINT technique to encapsulate siRNA in preformed PLGA nanoparticles followed by coating of the PLGA nanoparticles with lipids. The siRNA encapsulation efficiency was optimized in the range of 32-46% by varying the molecular weight or the lactic acid to glycolic acid ratio of the PLGA. They showed that the anti-Luc siRNA-loaded LPNs (200 nm) were highly effective in silencing luciferase gene expression in luciferase-expressing HeLa cells. Moreover, the LPNs were readily internalized in several prostate cancer cells

(*e.g.*, PC3, DU145) and in HeLa cervical cancer cells with minimal cytotoxicity. Subsequently, using a therapeutically relevant siRNA target (*i.e.*, KIF11), whose inhibition was known to cause mitotic arrest and apoptosis of cancer cells, they showed *in-vitro* that the siRNA-loaded LPNs successfully inhibited KIF11 expression in prostate cancer cells.

In addition to the DNA and siRNA delivery, LPNs have also been explored to deliver messenger RNA (mRNA) with the goal of developing mRNA-based vaccines. Using LPNs having biodegradable PBAE core and mRNA Su *et al*., [56] developed siRNA-mRNA complex. The *in-vitro* results showed that the LPM/mRNA complex was readily taken up by the dendritic cells – immune cells that play a key role in the initiation of adaptive immune responses. *In-vitro* and *in-vivo* results done afterwards showed affirmative results.

3.3. Diagnostic Imaging Agent Delivery: Owing to their high stability and superior bio-compatibility, LPNs are now being used as delivery vehicles of contrast agents in computed tomography (CT) and magnetic resonance imaging (MRI)s. For example, Mieszawska *et al*., [21] prepared LPNs carrying high payloads of gold nanocrystals (AuNC) and quantum dots (QD) by nanoprecipitation. He conjugsted AuNC and QD to the PLGA polymer by esterification before the lipid-polymer mixing step. The *in-vitro* results in mouse macrophage cells showed successful results.

Kandel *et al*., [57] developed LPNs having core made up of conjugated polymers having intrinsically high fluorescence (*e.g.*, PFBT) [58]. The LPNs (20–30 nm) prepared by nano-precipitation had PFBT nanoparticle core enveloped by lipid–PEG layer (*i.e.*, DMPE–PEG). Compared to the non-hybrid PFBT nanoparticles, the LPNs exhibited at least 50% higher quantum yields, therefore brighter fluorescence, which was attributed to the lipid tail insertion in the polymer core that created greater spacing between the polymer molecules resulting in intra-chain quenching, thereby higher quantum yields. In addition, the lipid–PEG layer also provided a tunable surface for LPNs functionalization. For example, when lipid–PEG with biotin-functionalized end group was used, the biotinylated LPNs effectively bound to streptavidin-conjugated beads, which was not observed with the non-hybrid PFBT nanoparticles. This signified the potential use of LPNs in specific labeling of biomolecules for bioimaging.

Furthermore, Kandel *et al*., [57] demonstrated that the biotinylated LPNs could be used for targeted labeling of specific cellular proteins, wherein this case the biotinylated LPNs were shown to be bound to biotinylated anti-CD16/32 antibodies on mouse macrophage cell surface receptors, using strep-tavidin as a linker. These results signified the PFBT LPNs as a viable technology for a wide range of labeling and imaging in living biological systems.

CONCLUSION AND PERSPECTIVES:

Preparation of LPNs: Till this date, with the application of advanced technologies and improved techniques a good control over LPNs desired size and physical characters are demonstrated. Though in theory the one-step method looks complicated, a majority of LPNs mentioned in this literature were prepared using this method. The one-step method (nanoprecipitation) is being increasingly preferred because it is simpler than the two-step one and faster than the later as well. Moreover, the physical characteristics of the LPNs prepared by the two-step method are dependent on the characteristics of the preformed lipid vesicles and thus there is less provision for a variation to produce better LPNs.

For the one-step method, between nano-precipitation and ESE, the nanoprecipitation method can produce smaller LPNs down to the sub-100 nm range, whereas the ESE method typically produces LPNs in size range of 200–300 nm. But the ESE method enables the encapsulation of a wider range of substances, regardless of their solubility and hydrophilicity, owing to the greater flexibility afforded by the emulsion formulations. Moreover, the ESE method generally results in higher encapsulation efficiency, hence content loading, than the nanoprecipitation. However, in terms of the recent developments, compared to the ESE method, the nanoprecipitation has advanced significantly further in the aspects of large-scale production capability and optimization of the LPNs' physical characteristics (*i.e.*, size homogeneity, uniform lipid coverage). These improvements were made possible by the development of a high-throughput continuous microfluidic nano-precipitation process.

Among the governing parameters, regardless of the method used, L/P ration has been found to have the most distinct effect on the LPNs size, colloidal stability, encapsulation efficiency, and release kinetics. However, optimizing the L/P ration lone is not sufficient for LPNs colloidal stability. So, in case of colloidal stability lipid-PEG fraction of the lipid formulation has been found to be the most important parameter because of the steric stabilization provided by the PEG chains.

The ESE method in itself has the upper hand over nanoprecipitation as the former can be used to encapsulate a far wider variety of substances. But in the recent future, developing a microfluidic process similar to the one for nanoprecipitation is of utmost importance as this will improve the LPNs size homogeneity and increase the production throughput of the ESE method.

In the longer term, as has been demonstrated with liposomes and polymeric nanoparticles the future research direction should demonstrate the feasibility of transforming the LPNs to the dry powder form, without causing significant adverse effects on the LPNs' physical characteristics. The dry-powder form is required for the LPNs for their stability during its shelf-life and hence better clinical acceptance.

Applications of LPNs: Only recently the application of LPNs have been explored in fields other than anti-cancer therapy; for example, for treatments of lung infection and injured vasculature, vaccine delivery, and bio-imaging. Most of the studies with LPNs involving non-cancer cells are in the preliminary stages where successful proof-of-concept has been demonstrated. But in case of cancer therapy, the superiority of LPNs over non-hybrid counterparts has long been proved. The use of LPNs in anti-cancer treatment have gone beyond single drug delivery. LPNs dual component structure provides an ideal platform for combinatorial drug delivery, whereby different drugs can be conjugated to either the lipid shell or the polymer core, depending on the desired release kinetics and the therapeutic roles of the drug in question. Application of LPNs' combinatorial drug delivery has successfully been demonstrated using drugs with genes, magnetic nanoparticles, and radio-isotopes. LPNs can further be targeted towards the site of action using different targeting moieties on their lipid shell depending on the desired site of action.

Though the advantages of using LPNs have been established in a number of in-vivo studies, a number of *in-vitro* studies are lacking. Hence, there is a need for studies demonstrating LPNs' superior delivery efficacy.

For the gene delivery application of the LPNs, the immediate future research need is clearly to demonstrate that the LPN/gene complex is capable of reducing the non-specific binding with serum proteins first *in-vitro*, then *in-vivo* upon systemic administration. After that is established, studies with therapeutically relevant genetic materials, instead of using reporter genes, will be needed for the research to move forward from the proof-of-concept stage to real therapeutic applications. For the bio-imaging application of the LPNs, the current body of work is still at its very early stage, such that additional studies are required before the benefits of having LPNs as the delivery vehicles of diagnostic imaging agents can be established with certainty.

To conclude, the LPNs development as therapeutic delivery vehicles has undergone tremendous progress since its inception. The tremendous progress is widely evident in demonstrative applications of the LPNs, where the benefits of having LPNs have been clearly established in vivo, particularly in drug delivery. The progress in the LPNs application, however, would be less impactful if it was not accompanied by improvement in the preparation method. Fortunately, the LPNs preparation method has also advanced significantly, where now simpler, yet better, methods capable of large-scale production have been developed, which is crucial for the LPNs to gain recognition beyond a laboratory setting. The future research needs that we have identified here will hopefully bring the LPNs closer toward the ultimate goal of its clinical realization.

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