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LIPID POLYMER-BASED BIO-INSPIRED DRUG DELIVERY SYSTEM

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ABSTRACT: Gene targeting has been a promising drug delivery system, thereby helping to improve the impaired functioning in various disorders. "Lipoplexes" and "Polyplexes" incorporates lipids and polymers combined with bioactive molecules, drugs, or genes are one of the newly developing systems. The need to protect genes from degradation, allowing them to reach their target site and to promote an efficient endosomal escape, to achieve this, the delivery system has been developed. Formulation studies and clinical trials are carried out on these systems, and they are found to be very useful in the treatment of various disorders such as Cancer, Parkinsonism, Cystic Fibrosis, Rheumatoid Arthritis, Antisense Therapy, and others. The major advantage of these systems over existing liposomal delivery is that they have gene silencing activity and are highly non-immunogenic & non-viral techniques of delivering genes to the target site. Encapsulating the drug within the lipids or the polymers and targeting them at the specific site reduces the non-specific reactions. The current review article focuses on the formation, mechanism of action, large-scale preparation, characterization, and applications of these lipoplexes and polyplexes that makes this system superior to other non-viral nanoparticulate drug delivery system.

INTRODUCTION: Conventional drug delivery systems such as oral, topical, or intravenous are easy to use yet are associated with a set of disadvantages. Some of them include higher doses, shorter half-life, degradation of the drug and adverse effects that in turn lead to toxicity [1]. The process of distributing a medication can have a prominent impact on its effectiveness. Most medications show an effect in the optimal range of concentration, and any increase or decrease in the concentration from the limit may be toxic or have no therapeutic benefits. Slow progress in the effectiveness of treating severe diseases has suggested the need for an integrative approach.

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New ideas were generated on controlling the drug's pharmacokinetics, pharmacodynamics, non-specific toxicity, immunogenicity, bio-recognition, and effectiveness.

Many such strategies rely on overcoming specific obstacles like the blood-brain barrier to better target the medication, enhance the efficacy, or on producing supplementary and appropriate ways to distribute protein drugs other than through the gastrointestinal tract, which might lead to its degradation. To overcome such barriers, researchers developed a new drug delivery system called lipoplexes and polyplexes. Lipoplexes refer to the use of lipids in combination with nucleic acid whereas polyplexes include the use of polymers enclosing nucleic acids in them. The particle size of these particulates was found to be between 1 to 100nm. (DNA, RNA, Proteins, and Peptides) [2].

Need: Liposomes, like some microparticulate delivery systems, have several disadvantages that

call for systems like lipoplexes and polyplexes to overcome their limitations. The disadvantages of the former system include conjugation with the components, reduced entrapment efficiency, use of non-biodegradable lipids, and many more. The sustained release period was found to be less as compared to the other systems. At times, the drug leaks out from the shell, thereby leading to fewer doses reaching the site of action, thus leads to a shorter half-life. During the preparation of the system, there are chances that the lipids might undergo chemical reactions like oxidation and hydrolysis. Liposomes are generally considered to be pharmacologically inactive [3]. Polymers on the other hand, are relatively cheap and can be synthetically prepared as per the requirement producing biodegradability and biocompatibility. Some major advantages of both lipoplexes and polyplexes are described below

Advantages: Targeting of the drugs, genes or any bioactive molecules is possible due to the characteristics of lipoplex and polyplex. Various advantages and disadvantages of these systems are mentioned below:

1. Increase in Drug Solubility: As the drug molecules are enclosed either in lipid or in a polymeric matrix, there is an increase in the solubility and also the bioavailability of poorly or partially soluble drugs.

2. Retard the Degradation of the Nucleic Acids and Cells: Entrapment of the drug in a lipid or polymeric matrix prevents its degradation from gastric fluids and other enzymes. This is also due to the diminution and the compression of the nucleic acid that helps to retard its degradation [4].

3. Long Circulation Time: PEGylation of the polymers or lipids enclosed with the drug improves the circulation of the drug in the body for a long period. It helps to decrease the particle-particle aggregation, particle size, and surface positive charge. This technique created small size lipids in the diameter range of 100-250 nm with an overall neutral charge. This in turn helped improve the pharmacokinetic properties [5].

4. Cell Targeting: As the particle size of these particles is around 200nm they undergo the "Enhanced Permeability and Retention" effect as a part of passive targeting. This is because the fenestration present in the RES system is about 100nm. [6]

Below are the advantages of both lipoplex and polyplex as a drug delivery system:

Lipoplexes: Lipids used in the preparation are either cationic, anionic, or non-ionic. Overall lipoplexes are non-immunogenic. They prevent degradation of the genetic material and causes destabilization thus, can easily escape from the endosomes. Cationic lipids can easily form complexes with nucleic acids as compared to anionic and neutral lipids. Anionic and neutral lipids act as secondary support to cationic lipids. These two types of lipids are usually used in combination to increase its efficiency and decrease the overall lipid concentration [4].

Polyplexes: Polyplexes employ the use of long branched polymers that are non-immunogenic and nonviral. The major advantage of polymers is the presence of a positively charged amine group that gets neutralized to form a compact structure with nucleic acids. The positively charged polymers tend to condense more DNA as compared to cationic lipids [4]. The synthetically prepared polymers are available in biodegradable forms to reduce its toxicity. Polymers can be tailored as per the size, in terms of concentration, composition and are liable to surface modifications [7]. Studies carried out suggest that the use of deionised polyplexes has shown excellent safety profile, stability, circulation half-life and thus can be used for tumor targeting or gene therapies *via* systemic administration [8]. They are proven to be good carriers for the delivery of siRNA as they provide simplicity in preparation and also found to be soluble in water thus providing better activity [9].

Disadvantages: Toxicity is majorly observed when the body identifies lipids or polymers as foreign, leading to hepatic or serological toxicity, immune responses, mutagenesis, and oncogenesis. Cationic lipoplexes in higher concentrations are toxic. Several inflammatory responses might also occur in patients receiving this therapy that might include either a rise in temperature, headache, myalgia, fatigue, *etc*. It is found that some lipids and polymers possess low transfection efficiency. Poly-ethyleneimine is a non-biodegradable polymer that

is still in use, thereby causing harmful effects. Experimentation concerning the size of the polymers reports that cytotoxicity is inversely related to size; thus smaller size polyplexes are responsible for causing apoptosis or cell necrosis [8]. The process parameters play an important role if they are not taken into consideration while formulating the system; there are chances that the delivery system might not be stable.

Theory: Lipoplexes and polyplexes can protect genetic material. As these lipids and polymers are cationic they firmly bind with the negative part of nucleic acids forming strong complexes with electrostatic interaction [10]. Studies illustrate the formation of these complexes and the factors that are involved in the formation, which include an increase in solubility with the use of polymers, temperature, concentration, and kinetics of mixing; environmental conditions also decrease in the degradation of the genetic materials and sensitive drugs.

Several theories state that both lipoplexes and polyplexes are effectively taken up by human cells and they work with the mechanism wherein first they are taken up via endocytosis, followed by apoptosis, also called programmed cell death. The other way is causing cytotoxicity, which can be done by modulating the activator gene which is responsible for causing cancer. These plexes will suppress the tumor cells and will block the entry of the nucleolytic enzyme [2]. The brief cellular mechanism and formation of these lipoplexes and polyplexes are explained below:

Formation of Lipoplex: Cationic lipids form charged complexes with the negatively charged DNA/RNA molecules (nucleic acids). These lipids are available in various shapes and sizes, which play an important role in the formation as well as during the entry at the cellular levels. Also, still, the effect of the particle size on the transfection efficiency, endosomal escape, and stability is under question. (check). Researchers suggest that the larger the size of particles (200-400nm) more is the efficiency and stability; however, others state that the lesser the size (200nm or less) greater the stability. Cationic lipids are generally associated with anionic and neutral lipids for the improvement in physical and physiologic stability [11].

Cellular Mechanism of Formation: The general mechanism of the formation of lipoplexes is a two-step process that involves DNA induction via membrane fusion and Liposome-induced DNA collapse. The latter leads to the formation of vesicles and thus attaches the DNA within the lipid moiety bilayer. This in turn, forms a quasispherical structure that has a particle diameter of 0.2mm.

The neutral complex thus formed is a string-like complex with a highly ordered multilamellar structure. Following are the steps that occur in the formation: -

1. Formation of Cationic Lipids: Lipids are classified into four major types: cationic, anionic, neutral, amphiphilic. They show a positively charged polar head and a non-polar tail that takes part in bilayer formation. The charges on the lipids are responsible for the electrostatic interaction with nucleic acid molecules. Lipids show their stability outside the cell. The disulfide bond is reduced by strong reducing groups inside the cell, leading to the collapse of the carrier–DNA complex, which enhances its release.

2. Self-assembly and Formation of Lipoplexes: The process of self-assembly is spontaneous. Application of isothermal titration calorimetry to complex formation between diC_{14}-amidine and DNA indicated two steps. The first step is an exothermic, rapid, and reversible reaction driven by electrostatic interactions, during which the liposomes are in their original shape. The second step is a slower, endothermic, and irreversible step involving the fusion and rearrangement of the liposomes.

3. Charge and Stability of the Lipoplexes: Cationic lipids form the lipoplexes easily but are unstable and highly toxic. A slight excess in positive charge leads to high transfection efficiency. Lipoplexes prepared at a certain positive/negative charge ratio may contain uncomplexed, free cationic liposomes. It is notable that plain liposomes and lipoplexes, in particular, are not at equilibrium but represent kinetically trapped systems. Hence, their structures are relatively stable upon dilution, whereas thermodynamically reversible systems such as micelles and microemulsions would immediately aggregate or disintegrate.

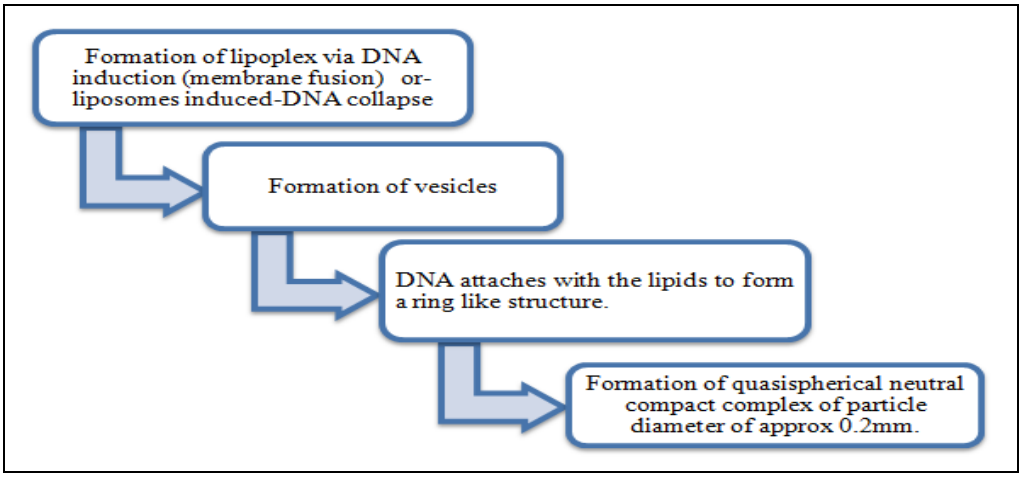


FIG. 1: FORMATION OF LIPOPLEX

Formulation Considerations: Several factors are responsible for altering gene delivery; a few are stated below:

1. Concentration of both Lipids and Polymers: An increase in the concentration of both lipids and polymers leads to toxicity, dose dumping, or serological inflammatory responses.

2. Temperature: An increase in temperature may lead to degradation of nucleic acids, leakage of the drug, and also melting of lipids and polymers.

3. Kinetics of Mixing: Mixing speed is also a major parameter to determine the formulation/formation of lipoplexes and polyplexes

4. Environmental Conditions: Chances of formulation degradation are more if the environmental factors are not taken into consideration.

5. Vector Structure: The shape, size, molecular weight, charge density, and stability plays an important role as it is associated with binding to the lipids and/or polymers [6].

Formation of Polyplexes: Polymers are found in various lengths and sizes thus; selection of the appropriate polymer is important so that it condenses with the nucleic acids. The condensation of these materials helps to form a stable and neutral structure showing efficacy and kinetic stability. Increasing the positive cation to negative, neutral acid ratio will increase the compaction and the efficacy of the formed product. Various studies suggest that a particular ionic strength as the formation of polycation is relatively faster than others and also permanent [8]. Factors such as transfection efficiency are affected by the way the substances are incorporated throughout the process. [12].

Intracellular Uptake: It is certainly difficult for the lipid-polymer particles to reach their target site and show their effect. Certain cellular endocytic uptake pathways are followed through which these complexes can enter the cell, further showing their action [13].

These carriers are destined to get degraded in the lysosomes or get effluxed out of the membrane. To prevent these certain pathways are followed that act as intermediates to protect the degradation, thus helping the drug to reach the target site. Endocytosis pathways include pinocytosis and phagocytosis, wherein pinocytosis is further divided into macropinocytosis, caveolae-mediated endocytosis, clathrin-mediated endocytosis and clathrin-independent endocytosis, as presented in **Fig. 2**. [14]

1. Phagocytosis: A process in which cells engulf large particles up to 20 µm. It is shown by specialized cells such as neutrophils, monocytes, and dendritic cells.

2. Pinocytosis: It is a generalized form of endocytosis, which involves the uptake of solutes along with extracellular fluids and is observed in all cells. Thus, depending upon the molecular mechanism of uptake, it is further divided into clathrin-mediated, macropinocytosis, caveolae-mediated, and clathrin-caveolae-independent; the latter three together forms clathrin-independent mechanisms.

3. Clathrin Independent Uptake Mechanism: They involve receptors, and membrane-associated proteins other than clathrin, such as caveolae, ARf6, Flotillin, CDC42, and RhoAthat helps in forming membrane vesicles for uptake of extracellular materials. Clathrin-mediated endocytosis acts like a hallmark for all cells. For instance, polarized epithelial cells lack caveolae on their apical membrane, whereas caveolae formation is prevalent in muscle, endothelium, and fibroblasts. These clathrin-independent mechanisms have been specifically used to enhance particle delivery to non-epithelial cells [13, 15].

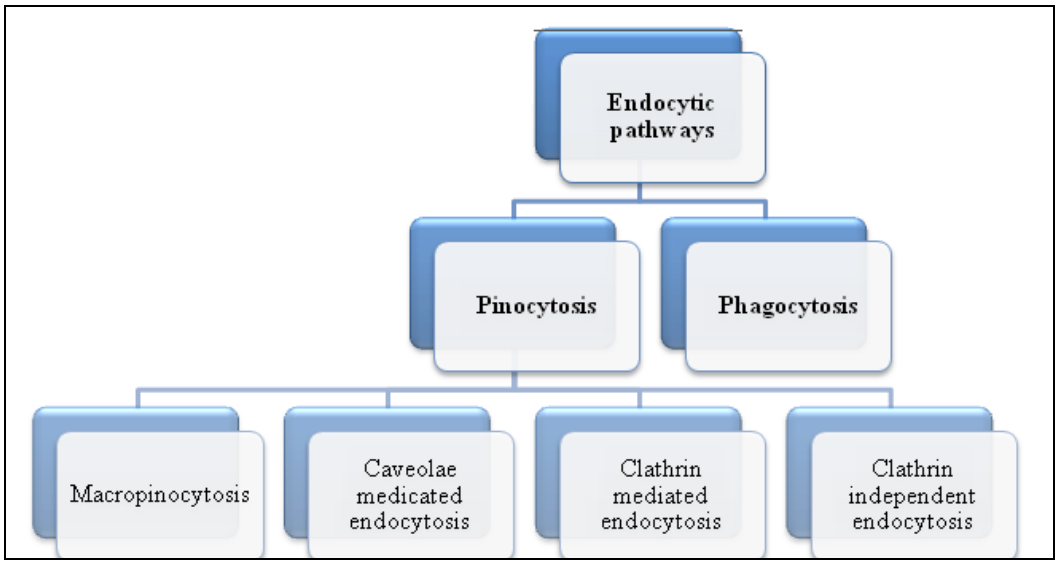


FIG. 2: ENDOCYTIC PATHWAYS

Flip-Flop Theory – by Lipoplexes: Flip flop theory states that lipids are positively charged whereas nucleic acids are negatively charged; thus, they interact via electrostatic interaction to form a plex. This plex then enters the cell membrane *via* endocytosis, thereby interacting with the charges of the transmembrane proteins of the biologic cell membrane, leaving an interaction with the lipids behind and forming a new bond of nucleic acid and biologic proteins showing this mechanism, destabilizing the endosomal membrane and releasing the nucleic acid into the targeted site which leads to gene expression [2, 16].

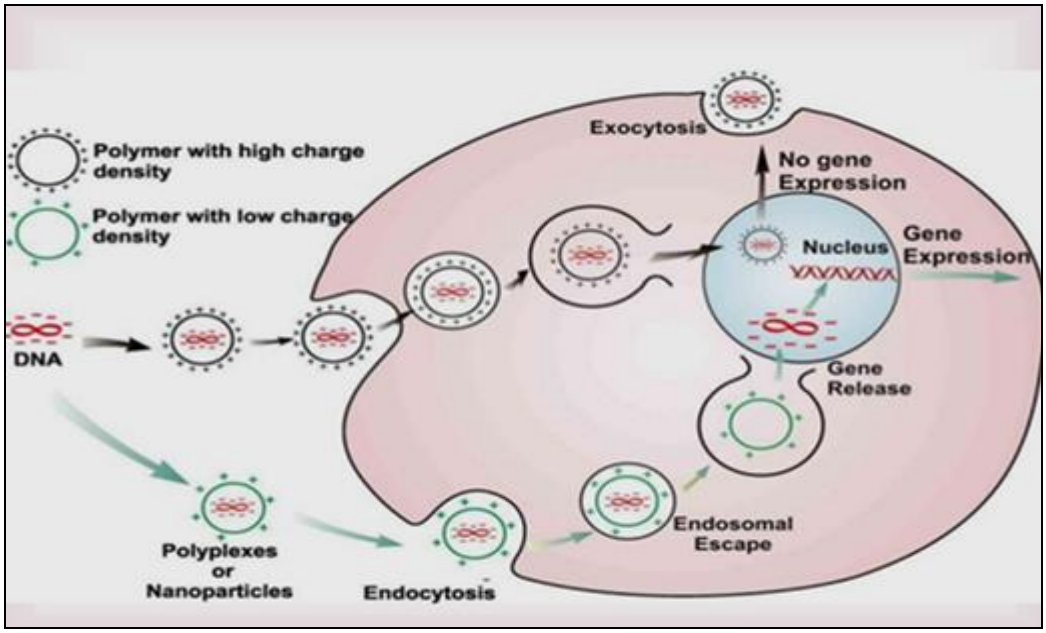


FIG. 3: FLIP FLOP THEORY

Proton Sponge Effect- by Polyplexes: It is important for DNA or any drugs that have been encapsulated in the polymer to elude out of the capsule to reach the nucleus; this process is called the endosomal escape. Some polymers can mediate this process with a unique mechanism called the "proton sponge hypothesis". The proton sponge effect states that once the polyplexes enter the endosomes, the presence of any weakly basic molecule will lead to an endosomal burst. The presence of the ATPase enzyme helps to actively transport protons from the cytoplasm to the endosomal vesicles. To balance out the effect of positively charged ions, there is an influx of negatively charged chloride ions to stabilize the charges and make the compound stable. These show high buffering capacity, thus a reduction in pH. Due to differences in the osmotic pressure, ionic strength within the cell, there is an endosomal burst with the release of polyplexes into the cytosol, which is known as the Proton Sponge Effect [2, 17].

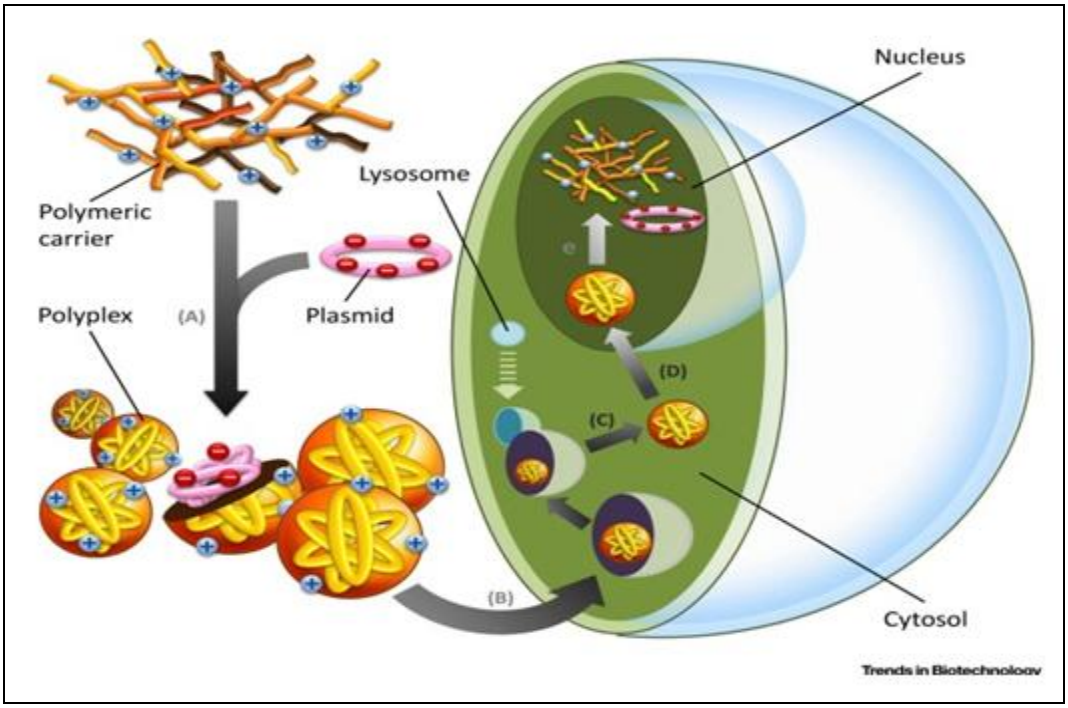


FIG. 4 PROTON SPONGE EFFECT

Materials: The raw materials utilized in the preparation of lipoplexes and polyplexes are stated below:

1. Nucleic Acids: These are either proteins or peptides, synthetically prepared DNA or RNA molecules, siRNA that have specific targeting actions or cytotoxic effects that, when delivered to the site of action, show their therapeutic effects. Few examples of vectors that are used are Bcl-2-siRNA, Choline Kinase siRNA, Mcl 1 siRNA, Endostatin yeast gene. Vectors can also be used as a part of gene delivery. For example, poxvirus, adeno-associated virus, retrovirus, and others [18, 19].

2. Drugs: Active pharmaceutical ingredients such as doxorubicin, rituximab, trastuzumab, curcumin, *etc*. shows anticancer activity, some of which are used in the treatment of breast cancer. These drugs are encapsulated in the core of the lipids or the polymers which are then delivered to the target site [20].

3. Surfactants: The main purpose of surfactants is to reduce the interfacial tension between the molecules, helping in providing better permeation of the particles into biological fluids. They are also helpful in maintaining the stability of the final formulation. Examples of few surfactants used are Sugars, Tween 80, Gemini surfactants [21, 22].

4. Organic Solvents: They are required in the manufacturing processes to act as a volatile solvent that initiates mixing and, in turn, leaves a film or complex with itself getting evaporated. Some of the majorly used organic solvents are ethanol, chloroform, di-methyl-sulfoxide (DMSO), *etc*. [23]

5. Vehicle: They are used for dissolution of active ingredients that may or may not be functional [24].

6. Lipids: Lipids are one of the most important components used in preparation. They are used as an encapsulating agent because of their long hydrocarbon chain that helps in penetration of the drug. Majorly those lipids that resemble the structural features of the body components are used so that the permeation of the vesicles is easy, thus avoiding the reticuloendothelial system [25]. Based on their structural properties, they are further classified into 4 types: cationic, anionic, neutral, and ionizable [11].

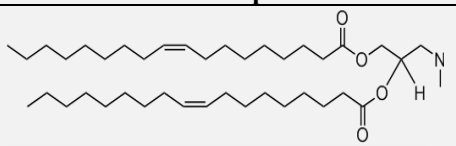
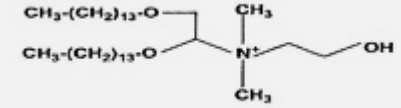
i. **Cationic Lipids:** They are amphiphilic made up of positively charged polar groups and hydrophobic domains with chain lengths between 8 to 18 carbons. The head group is responsible for the transfection of the lipoplex; it also contains an amine group with substitutions such as guanidine, pyridinium, and other peptides. The major disadvantage is that they are toxic; any modifications in the head group, the linker, or the hydrophobic tail can help to regulate the cytotoxic levels. Some examples of lipids are (N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammoniumchloride)-DOTMA, Dimethyl-ocyadecylammonium bromide –DDAB *etc*. [26]

ii. **Anionic and Neutral Lipids:** The anionic and neutral lipids are majorly used as secondary support or as co-lipids because of their low toxicity. Some examples are phosphatidylglycerol, phosphatidylserine. The majorly used ones are Cholesterol, Dioleyl phosphatidylcholine (DOPC), Dioleyl phosphatidylethanolamine (DOPE). These lipids are also found to improve the carriers' efficiency of lipoplexes. The possibilities of electrostatic interactions of lipids with charges of the vectors are less; Thus, there comes a need for complexation ions such as Ca^{2+}, Na^{+}, Mg^{2+} [27, 28].

iii. **Ionizable Lipids:** They can self-assemble when mixed with nucleic acids. They have also been found to improve gene delivery efficiency [29]. The descriptive examples of lipids are mentioned in the table below:

7. Polymers: They have long-chain flexible structures which are either linear or branched with positive charges that encapsulate the negatively charged nucleic acids in them. Cationic polymers are mostly used in formation that includes polylysine, poly-arginine, and cationic dendrimers like polyamidoamine (PAMAM), carbohydrate base- Chitosan. Synthetic polymers or the first-generation polymers show low buffering capacity & transfection efficiency and also show toxic effects which are modified by PEGylation, functionalization with other substitutes to minimize their cytotoxic effects [11, 30]. The polymers need to be non-viral, non-immunogenic. Biocompatibility is the most important characteristic required in a polymer so that lipoplexes like nanoparticles are not removed by the reticuloendothelial system [31].

TABLE 1: EXAMPLES OF LIPIDS

Name	Structure	Description	Ref
Cationic lipids			
Dioleoyl-3-tri-methyl-ammoniumpropane(DOTAP)		It produces highly efficient transfection and also be successfully used for in vivo applications.	32
1,2-dimyristyloxypropyl-3-dimethyl-hydroxy-ethyl-ammonium-bromide(DMRIE)		It shows high transfection efficiency in case of In-vitro and In -vivo	33
1,2-di-o-octadecenyl-3-trimethylammonium propane (DOTMA)		DOTMA is a cationic lipid and a non-viral vector for gene therapy, also a building block of the siRNA delivery system	34
Anionic lipids			
1,2-dioleoyl-sn-glycero-3-		They tend to orient themselves in the	35

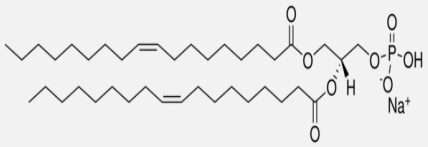
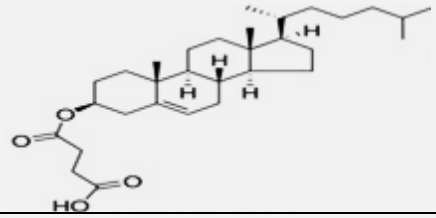
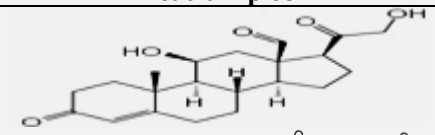
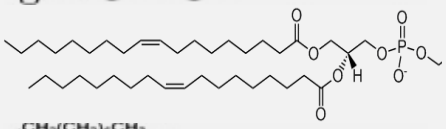
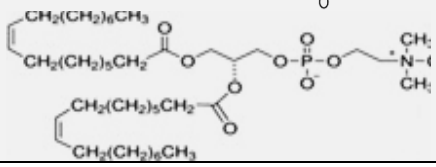
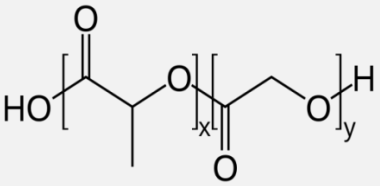
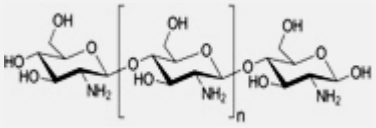
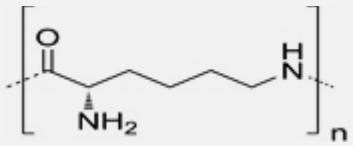
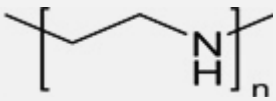
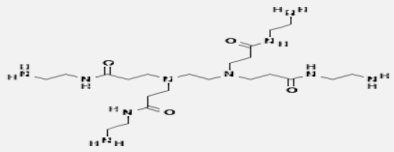
phosphate (DOPA)		presence of solvents.	
Cholesteryl Hemi-succinate (CHEMS)		It self-assembles into bilayers in alkaline, neutral ,aqueous media and is employed in mixtureswithdi-oleoyl-phosphatidyl-ethanolamine (DOPE) to form 'pH sensitive' fusogenic vesicles	36
Neutral lipids			
Cholesterol		Enhance lipophilicity thus increases permeation and enhances drug delivery.	4
Di-oleyl-phosphatidyl-ethanol-amine (DOPE)		Increases transfection, increases cellular uptake	37
1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC)		They are the most abundant lipid in the human cell providing a structural framework and acts as a permeability barrier.	38

TABLE 2: EXAMPLES OF POLYMERS

Name	Structure	Description	References
Poly-Lactic co- Glycolic Acid (PLGA)		PLGA is one of the polymers that are most widely used asdelivery carriers for controlled administration of drugs, peptides, and proteins due to its biocompatibility and biodegradability property.	31
Chitosan		It is a highly compatible, biodegradable, less immunogenic and can easily form complexes and is degraded in the body due to lysozymes	39
Poly-l- lysine (all)		This polymer is biodegradable, but lack transfection efficiency. It is capable of forms water-soluble complexes with negatively charged macromolecules	40
Polyethyleneimine (PEI)		It is high transfection efficiency and is also biocompatible	41
Poly-caprolactone (PCL)		They are used in the preparation of microcapsules and nanoparticles because of their structural flexibility. In the case of nanoparticles, they tend to improve the photochemical stability	42
Poly-amido-amine (PAMAM)		It is a hyper branched polymer with repetitive amine units. It is used as vectors for the delivery of drugs in breast cancer	43

Methods of Preparation: The delivery system can be prepared using 3 methods which are as follows:

1. Direct Mixing
2. Hydration Mechanism
3. Microfluidics

A. Direct Mixing:

1. This technique involves easy lab to large-scale methods for the preparation of lipoplexes. They can be done in 3 ways which are demonstrated in **Fig. 6**.

2. The first is the conventional method or the standard method of liposome preparation which involves the dissolution of the lipoid system in chloroform or any other organic solvent, which is then evaporated to create a thin film. This film is then hydrated, which leads to the aggregation of the particle, which is later mixed with DNA.

3. The second is the "Simple Injection" Liposome preparation: this involves the vortexing of a two-phase system wherein one phase is lipid dissolved in an organic solvent like ethanol and water being the other phase. This whole system is then mixed with DNA [44].

4. The third method involves “Direct Mixing” of the genetic material in the solution of lipids.

5. The following example helps to understand the process better [45] prepared lipoplexes using the 'Ethanol Injection' method, which involved the dissolution of lipids in absolute ethanol in a round bottom flask (RBF) at about 60°C.

   Preheated water was added to the above mixture, which resulted in a milky suspension. The ethanol is then removed by a rotary evaporator under reduced pressure with the passage of nitrogen gas. Again, when water was added, the mixture turned transparent; this initiated the formation of liposomes.

6. Maitani, 2010 also prepared lipoplex using the 'Direct mixing" method wherein a ratio of 1:11 lipid to DNA was formed by the addition of 1-3.73mg total lipid/ml water and 1 microgram of DNA with gentle shaking. DC-Chol/DOPE was the lipids used in the preparation of lipoplexes, and it was found that this method involves smaller lipoplexes as compared to the above methods [45].

7. Meisel & Gokel performed this direct mixing method wherein the lipids are dissolved in dimethylsulfoxide (DMSO) or ethanol (EtOH) which is directly added to the aqueous nucleic acid solution.

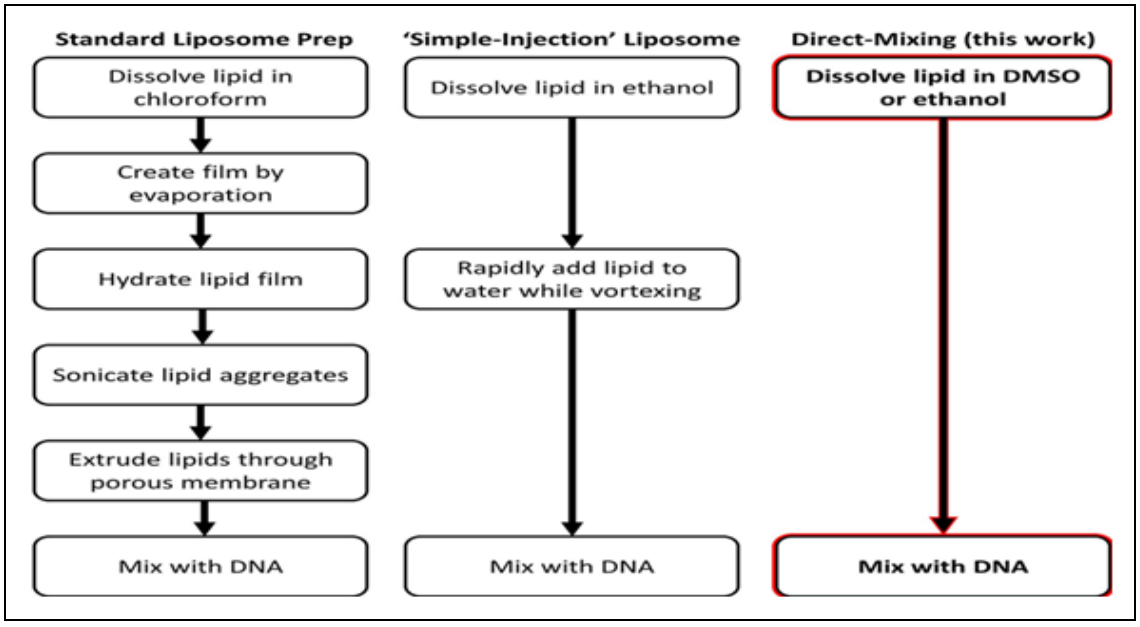


FIG. 5: FLOW CHART OF DIRECT MIXING

8. The researchers thus concluded that the method is fast, simple, efficient, and does not require any specialized instruments, also enables quick optimization of the batches leading to faster analysis.

9. The study also states that the transfection efficiency of these lipoplexes was similar as compared to the methods used for the preparation of liposomes but the toxicity of these lipoplexes prepared using DMSO and Ethanol observed was less as compared to the liposomal method [23].

B. Hydration Mechanism – Rotary Evaporator:

i. This is again a lab-scale method that involves the use of the instrument called the 'rotary evaporator' for the formation of vesicles

ii. The vesicles so formed from this method are of different shapes and sizes that include small unilamellar, large unilamellar and multilamellar vesicles. The process involves the following steps: mixing the lipid in the organic solvent for its solubilization, then evaporating the liquid under vacuum in the RBF, leaving a dry lipid film. The addition of any hydrophilic drugs in a water solution this step is called Hydration. The mixture is stirred, which gives rise to multilamellar vesicles. Following sonication, extrusion, homogenization, and micro fluidization, we get small unilamellar vesicles and large unilamellar vesicles (LUV's). The final mixture containing LUV's and SUV's are centrifuged, dialyzed and ultra-filtered to obtain the final preparation. Cationic and Neutral lipid are complexed in the ratio of 1:1. The RBF contains chloroform: methanol 4:1 v/v. The solvent is then evaporated, and the remaining mixture was disseminated & extruded through a membrane of pore size 0.1nm. [46, 47]

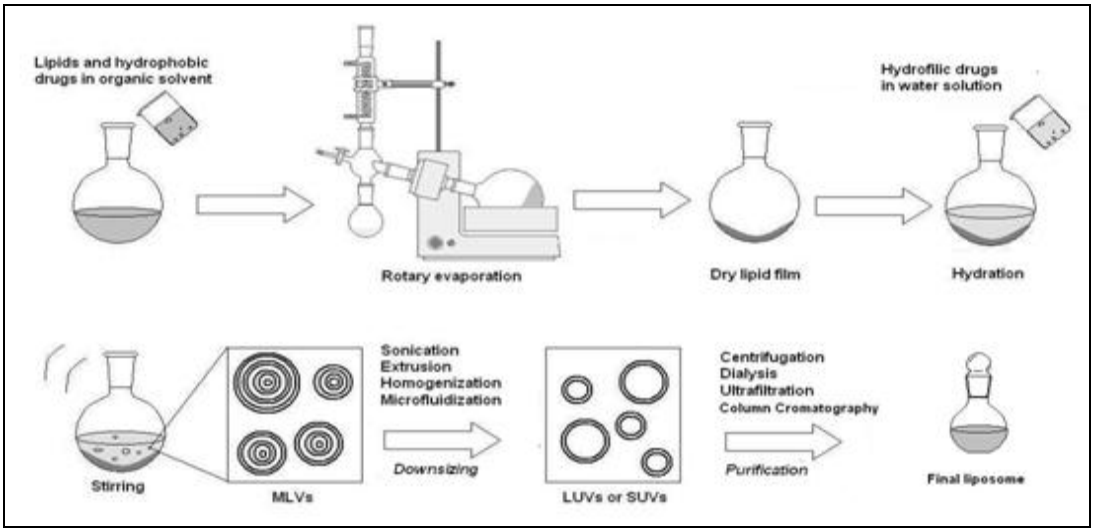


FIG. 6: HYDRATION MECHANISM BY ROTARY EVAPORATOR

C. Micro Fluidics Technology:

i. Microfluidics is one of the finest new emerging technologies being used in the formulation of lipoplexes and polyplexes like the delivery system. The process involves the use of the design of experiments and multivariate data analysis with predictive models. The technology refers to fluid handling in controlled volume mostly below the milliliter scale. This is a novel lab on a chip-based device that reduces the sample preparation time and helps to generate robust and reproducible results.

ii. Method: It involves the use of flow focusing 'T' or 'Y' shaped mixers. The lipoid and aqueous mediums are dissolved and incorporated in the inlet of the microfluidic cartridge. The precipitation reaction is

responsible for the formation of nanoparticles. The classic example explained here is the use of DOPE and DOTAP as the fusogenic lipids, which were dissolved in ethanol and were passed through the inlet with the simultaneous addition of aqueous buffer in the mixer. The flow rate and flow ratios were monitored. The resulting lipoplexes were then characterized for its entrapment efficiency, cytotoxicity, uniformity of content, and in vitro transfection. The Hydrodynamic Flow focusing method is projected here:

iii. Hydrodynamic Flow Focusing: In contrast to the conventional method of novel synthesis, nowadays 3D- Hydrodynamic flow-focusing method, which is a part of microfluidic technology is used that leads to the formation of robust, reproducible results with smaller size particles and reduced cytotoxicity. The example here explains the formation of polyplex by adding the DNA into the polymeric solution, which was then subjected to high vortexing of the solution. Electrostatic interactions between the cationic polymer and the anionic DNA led to this system. The use of a ratio of the formed system was then subjected to various attributes like flow rate, zeta potential, flow ratio. etc. It was found that an increase and decrease in the flow rate have its effects on the size of the polymer with DNA. Also, it suggests faster the mixing smaller the polyplex generated. Thus the 3D-hydrodynamic flow-focusing method helped develop smaller size plexes with slow aggregation rate and low cytotoxicity [48, 49].

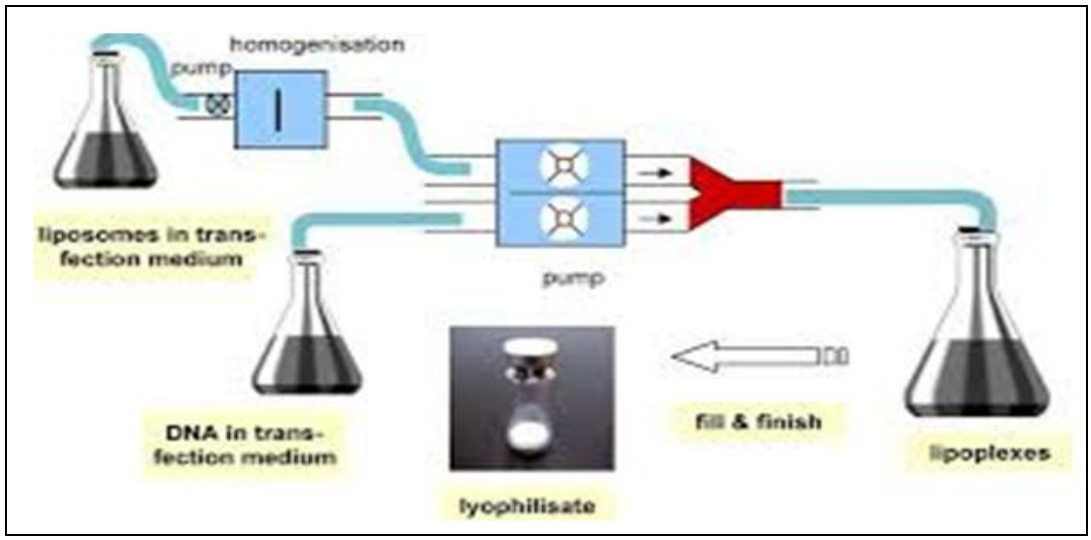


FIG. 7: MICRO FLUIDIC TECHNOLOGY

Characterization: The structure, size, shape, formation, the concentration of lipids & polymers, and other such factors play an important role in understanding the safety and efficacy of the formed particles. Some of the characterization techniques, along with the parameters, are listed below in **Table 3**.

TABLE 3: CHARACTERIZATION TECHNIQUES

S. no.	Instruments	Parameters	Ref
1	Gel Permeation chromatography	To determine the cloud point	8
2	Gel Retardation Assay	Helps to quantify free siRNA	9
3	Electron Spin Resonance	Determines the motion & structure-based features	12
4	Super-Resolution Imaging	To study the nanocarrier cell interactions	14
5	Evaporating light scattering	To determine the concentration of lipids and polymers with Nonviral vectors	50
6	FTIR spectroscopy	Measurement of cationic lipid & polymers. And the determination of the functional group	51

7	Scanning electron microscope	Characterize the lipoplexes & polyplexes	[52]
8	Circular dichroism Spectroscopy	To study the alteration to secondary structure and conformation adopted by biomolecules under various conditions	[53]
9	Dynamic light scattering	Measurement of zeta potential	[54]
10	Confocal laser scanning microscopy	Identification of the fluorescently labelled particles	[55]
11	Fluorescence Cross-Correlation Spectroscopy	Quantitative determination of the extent of association of the lipids and the DNA along with the loading capacity	[56]
12	Agarose Gel Electrophoresis	To determine the formation of polyplexes	[57]
13	Static Light Scattering	To measure the gyration and hydrodynamic radius of polyplex	[58]
14	Small-angle X-ray Scattering	Investigation of lipid conformation	[59]
15	Molecular Modelling	Helps in the estimation of size and charge density of polycations	[60]
16	Transmission-Electron Microscope	Structural characterization of formed lipoplexes and polyplexes	[61]
17	Cryo-TEM Analysis	Structural characterization of synthetic vectors	[62]
18	Nuclear Magnetic Resonance	To determine the structural properties	[63]

Applications:

1. Treatment of Colorectal Cancer:

i. Shi *et al.* selected two pro-drugs that were covalently linked to each other *via* ϒ-glutamyl-cysteinyl glycine sensitive linkage and paclitaxel with mPEG-PLA-COOH through reactive oxygen species sensitive-linker. The nanoparticulate systems were formulated by evaporating the solvent or emulsion and were further modified with peptides. The physicochemical properties of nanoparticles were determined by TEM analysis. *In-vivo* tumor targeting and anti-tumor assay were investigated on the colorectal cancer-bearing mice. Paclitaxel and ϒ- glutamylcysteinyl glycine coated with Polyethylene Glycol, was proven to show antitumor effect and was enough for targeting the tumor cells [64].

ii. Ho *et al.*, studied the antiangiogenic fusion protein RBDV-IGg, Fc with receptor binding domain of vascular endothelial growth factor along with polyethyleneimine and polyethylene glycol to target tumor cells. The lipoplex-poly-PEG-PEI complex was successful in protecting the DNAse; it could complex with the VEGFR cells both In-vitro and *in-vivo.*

These were also capable of suppressing the tumor growth in-vivo thereby providing better therapeutic efficacy [65].

iii. A report generated from phase 2 clinical trial, which included 27 patients were given infusions of ONYX-015 along with 5-fluorouracil. Overall, the anti-tumoral effects could not be assessed in patients who were given the same regimen earlier but, 3 patients had 30-50% regression of tumor mass [66].

2. Treatment of Breast Cancer: The drug Herceptin (Trastuzumab) along with curcumin was used to target HER2 receptor. The combination of cationic liposome-PEG-PEI was used as a carrier to achieve cell targeting. This combination was found to be anti-inflammatory, anti-tumor, and anti-metastatic. The drug-loaded with lipopolyplex complex showed cytotoxic activity in the cancerous cells. Final formulations of drug-loaded lipid polymer complexes were compared with the drug treatment alone, which resulted in better therapeutic efficacy. The results showed that lipoplex was capable of reducing tumor from 75% to 55% thus increasing the life span of the mice [67].

3. Treatment of Ovarian Cancer: Over expression of folate receptor α and high telomerase activity are characteristics of ovarian cancer. The use of human telomerase reverse transcriptase is a tumor-specific promoter and can direct target gene expression in human ovarian cancer cells. The formulation was prepared with PEG into folate modified liposome along with plasmid to form a lipoplex and the drug-loaded lipoplex showed anti-

tumor activity, thereby reducing the number of cancerous cells [68].

4. Treatment of Lung Cancer:

i. Lung cancer is by far found to be a leading cause of cancer-related deaths. SOX2 gene has shown to be a hallmark for lung cancer and is responsible for the formation and progression. Andey *et al.,* formulated lipoplexes with the help of small interfering SOX2 gene to study protein expression, cell viability, and efficacy. The results concluded that there was an increase in the cell viability with several folds, high drug loading capacity and were also capable to induce tumor regression [69].

ii. miR-133b are noncoding RNA's that are responsible in several biologic processes such as proliferation, apoptosis, and development. This gene also functions as biomarker for the diagnosis and prognosis of lung cancer. miRNA was formulated as lipoplexes *via* ethanol injection method used to target gene MCL-1 thus regulating cell survival and sensitivity of lung cancer cells to chemotherapeutic agents. The efficacy of these particles did not show any cytotoxic effects. All these advantages showed greater potential for tumor targeting in lungs [70].

5. Treatment of Liver Tumor: Liver cancer is one of the most frequently diagnosed cancers. A study was developed as an attempt to use doxycycline coated with a gene to improve drug targeting and decrease systemic side effects associated with doxorubicin. Lipoplexes using poly-γ-glutamic acid (γ-PGA)-coated Doxorubicin were developed to enhance anti-tumor activity to treat the liver tumor. Cell lines studies were carried out to determine the efficiency and efficacy of the formed lipoplexes. Along with cell line study, cell apoptosis was determined by flow cytometry. The results of cell line studies suggested that the formed lipoplexes were able to inhibit the growth of HepG2 cells. Results of apoptosis studies concluded that PGA-L-Dox-loaded lipoplexes increased the apoptosis rate of tumor gene. Overall, these particles showed high cellular uptake, *in-vitro*-cytotoxicity, cell apoptosis and exhibited *in-vivo* anti tumor efficacy [71].

6. Treatment of Parkinsonism: Trojan Horse Liposome is an immunoliposome alternative to gene delivery. DNA encapsulated in the internal cavity of liposomes with lipids such as PEG prolong the circulation time in blood. These particles protected the DNA from nuclease degradation. The lipoparticles were able to mediate transcytosis across the blood-brain barrier [BBB]. They were able to normalize the striatal tyrosine hydroxylase activity. The studies also suggest that lipopolyplex system for Parkinsonism has higher gene transfection efficiency than lipoplex [72, 73].

7. Treatment of siRNA for Melanoma Therapy: Dorrani *et al.,* explains that siRNA has a potential application in dermatological diseases. A series of liposomes preparation were prepared to efficiently deposit these particles in the basal epidermis where the melanoma cells reside. The lipoplexes and liposomes were formed, and the efficacy was compared. Skin permeation studies were carried out using fluorescently labelled particles. The results of the study suggested that lipoplexes were able to enhance the skin permeation and also knock the expression of tumor genes [19, 55].

8. Treatment of Rheumatoid Arthritis: Rheumatoid Arthritis is an autoimmune disorder of the joint. Non-steroidal anti-inflammatory drugs is the first-line therapy used for its treatment. Newly emerging treatment strategies include gene therapy that has the potential to avoid any side effects associated with the current therapies available. The use of complementary DNA (cDNA), siRNA, non-viral vectors coupled with polymers or lipids are showing the ability to suppress TNFα and progression of arthritis in experimental models. Young *et al.,* along with his group of scientists, made an attempt to inhibit pro-inflammatory cytokines using naturally occurring antagonists.

For example, IL- Ra, is an endogenous antagonist of IL-1, which is normally upregulated during rheumatoid arthritis. The scientists carried out an *ex-vivo* study which was one of the first gene therapy to reach clinical trials. The fibroblasts were extracted from the patient's joints, and it was transduced with IL-1 cDNA with the help of retroviral vector. The patient showed symptomatic improvement during the trial [74].

9. As Gene Silencer:

i. Conejos-Sánchez *et al*., formulated poly-ornithine-based polyplexes for delivery of siRNA for the treatment of Multiple Sclerosis. Neural cells were targeted to evaluate the gene silencing efficacy. Gel permeation chromatography, Diffusion ordered NMR spectroscopy was carried out to determine the diffusion coefficients. The formed polyplexes provided sustained transfection efficiency.

These also exhibited silencing of luciferase gene expression. Other studies such as Protein expression analysis, Gene Expression through Real-Time qPCR, Immunofluorescent studies were carried out. The formed polyornithine polyplexes showed good stability, gene silencing expression, and transfection efficiency, proving to be a better alternative for treating Multiple Sclerosis and other kinds of CNS disorders [53].

ii. siRNA is also known as small interfering RNA has the potential to provide gene silencing, enhance the sensitivity of tumor cells towards chemotherapeutic agents. Y. Wang *et al.*, formulated a novel siRNA lipo-polyplex that was modified with peptides for epidermal growth factor receptor-targeted delivery into EGFR positive tumor cells. Gene silencing activity in the receptors was investigated against siGFP, which is a green fluorescent protein that targets siGFP-luci-ferase fusion stability.

Further, the formed polyplexes were characterized for their cellular internalization and functionalization, anti-tumoral activity, and cell cycle analysis. Researchers concluded that siRNA poly-plexes formed with lipo-oligoamino peptides with 8 cationizablesuccinoyl-tetraethylene-pentamine (Stp) units were far more stable than those formed by containing only four Stp units; incorporation of histidine exhibited higher gene silencing efficiency. Polyplexes made of PEG-GE11 demonstrated superior cellular uptake.

All these were verified by the highest antitumoral potency tumor cell lines [75].

10. As a drug carrier:

i. The lipids and polymers act as an encapsulating material for those responsible to degradation, thus improving their bioavailability and pharmacokinetic properties. They protect the genetic material and target them to the specific site, thereby reducing adverse effects [11].

ii. They are also used in formulating drugs for the treatment of diseases like haemophilia, asthma, cystic fibrosis [76].

iii. Studies suggest that they can be used in the delivery of flavonoids like hesperidin as an anticancer prodrug, that can act as an alternative to chemotherapy [77].

CONCLUSION: "Lipoplexes" and "Polyplexes" are systems that incorporate lipids and polymers combined with bioactive molecules, drugs, or genes. Targeted gene delivery *via* lipoplexes and polyplexes has shown a colossal advancement for the delivery of bioactive drugs and viral vectors. They impart properties like low cytotoxicity, high entrapment efficiency, non-immunogenic virogen transfer, and high transfection efficiency.

These formulations also possess to be less toxic, easy to manufacture and characterize as compared to other nanoparticulate formulations. Other parameters like prevention of degradation *via* DNAse make them superior as a drug delivery system. The future perspective of lipid and polymer-based bio-inspired drug delivery systems associated with nanotechnology seems to be a promising approach in the treatment of various disorders.

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