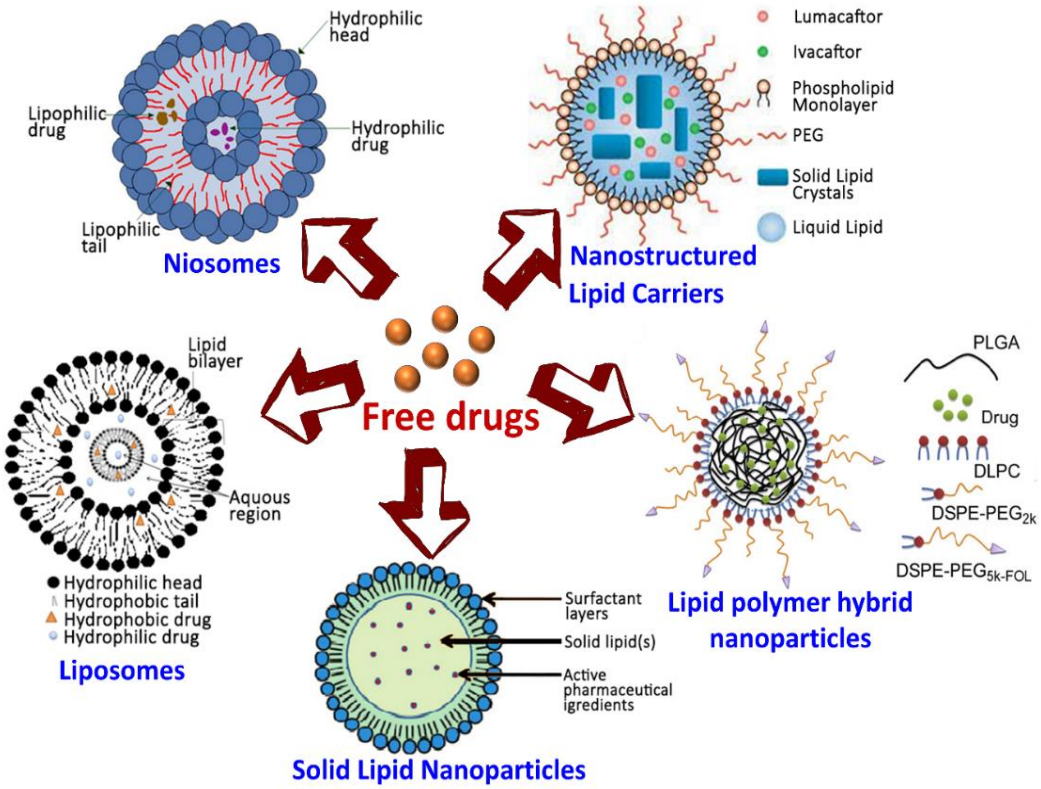
Chapter I

Introduction and Review of literature



Rahul Kumar et al., ***Journal of drug delivery science and technology.*** *74(2022)103525. doi.org/10.1016/j.jddst.2022.103526.*

Rahul Kumar et al., ***Pre-print, Authorea, Netherland.*** *doi:10.22541/au.164605916.66670168/v1.*

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Candidate CRediT and collaborators contribution statement

Rahul Kumar: Data curation, formal analysis, investigation, formal analysis, writing – thesis, review & editing, and conceptualization.

Collaborators: Review & editing, resource, validation, and software.

1. General introduction

In view of drug delivery, diverse delivery vehicles including natural and synthetic polymeric nanoparticles have been greatly investigated till date. In general, micelles, dendrimers, cellulose, gelatin, lipid, chitosan, alginate, poly (D,L-lactide),poly (D,L-glycoside), poly(lactide-co-glycoside), and poly-caprolactone have been employed as drug delivery vehicles .However, we are interested to select lipid based nanoparticle/nanocarrier for targeted anticancer drug delivery. Since then, nano lipid-based carriers (NLBCs) have emerged as the most facile and efficient vehicle to overcome the physicochemical and physiological barrier. At the beginning of 21st century, NLBCs have taken advantage of conventional targeting for therapeutic delivery in terms of better biocompatibility, intrinsic penetrating capacity, easy fabrication, and non-toxicity **[1]**. The various attributes associated with NLBCs include drug protection, enhanced bioavailability, reduced doses, improved treatment, enhanced oral bioavailability, and surface modification flexibility. Additionally, NLBC's can precisely transport both hydrophilic and hydrophobic medicines, resulting in improved therapeutic efficiency **[1]**. NLBCs have a high surface area that can be modified with various chemical moieties such as polyethylene glycol, maleimide, poly-amino acid, carbohydrates, and fatty acids to surpass the reticuloendothelial system. Moreover, they provide platforms for conjugating the targeted ligand through various chemical reactions including EDC-NHS cross-linking for specific receptor detection and diagnosis **[2,3,4,5].** Incorporating bioactive compounds into comparatively biocompatible inert carriers and site-specific targeting using specific ligands represents a promising strategy for increasing its therapeutic index while lowering the side effects **[6,7].**

Cancer is an unregulated and uncontrolled cell division that emerges due to genetic or epigenetic changes which alter the normal signaling pathway **[8,9]**. Whereas, cancer metastasis is a series of events that result in the production of secondary tumors in distant

organs and is mostly responsible for cancer mortality and morbidity. It has been reported as the second-leading cause of mortality among other diseases, and incidences and fatality rates are expected to rise drastically in the coming years. According to the world health organization (WHO) report, a total of 19.9 million people were diagnosed with cancer in 2020. However, 10 million people died of cancer in the same year. Among total incidences of cancer, Asia shared maximum percentage of cases (49.3%) followed by Europe (22.8%), Northern America (13.3 %), Latin america of the Caribbean (7.6%), and Africa (5.7%.). Amidst mortality, Asia also stood first and shared 58.3% of total cases followed by Europe 19.6%, Latin America of the Caribbean (7.2%), Africa (7.1%), and North America (7.0%). Breast cancer solely accounts for 11.7% of all the newly diagnosed malignancies in women. This indicates that in the world, one out of every eleven newly diagnosed cancers in women are breast cancer. However, lung cancer was responsible for almost 18% percent of all cancer-related fatalities worldwide among women. Therefore, in view of cancer burden worldwide, we have targeted the cancer cells for drug delivery. At present, chemotherapy, radiotherapy, and surgery are the only alternatives to treat cancer, depending upon the nature and position of the tumor. These treatments are only limited to localized tumors and not metastatic tumors resulting in the normal cells getting affected, leading to a high recurrence rate and death. Chemotherapy, utilizing strong chemicals, has unfavorable effects on healthy cells and tissues due to a lack of specificity and cannot differentiate between normal and cancerous cells **[10]**. Also, drugs alone cause substantial issues such as poor absorption, solubility, bioavailability, high degradation rate, short shelf-life, and low therapeutic index **[11]**. Multiple drug resistance (MDR) has emerged as a key stumbling block in cancer treatment in recent years. Therefore, the above limitations can be encountered by using the concept of theranostics along with nanocarriers. Theranostics is a combination of diagnostic (transferrin, protein/peptide, folic acid, aptamer, and carbohydrate) and therapeutic agents (nucleic acid, drug, and vaccine) **Figure 1.1**. We conducted a scientific

survey related to 'lipid nanocarrier and cancer theranostics' through the online database 'Scopus'. The survey results suggested tremendous growth in the last ten years **(Figure 1.2)**.

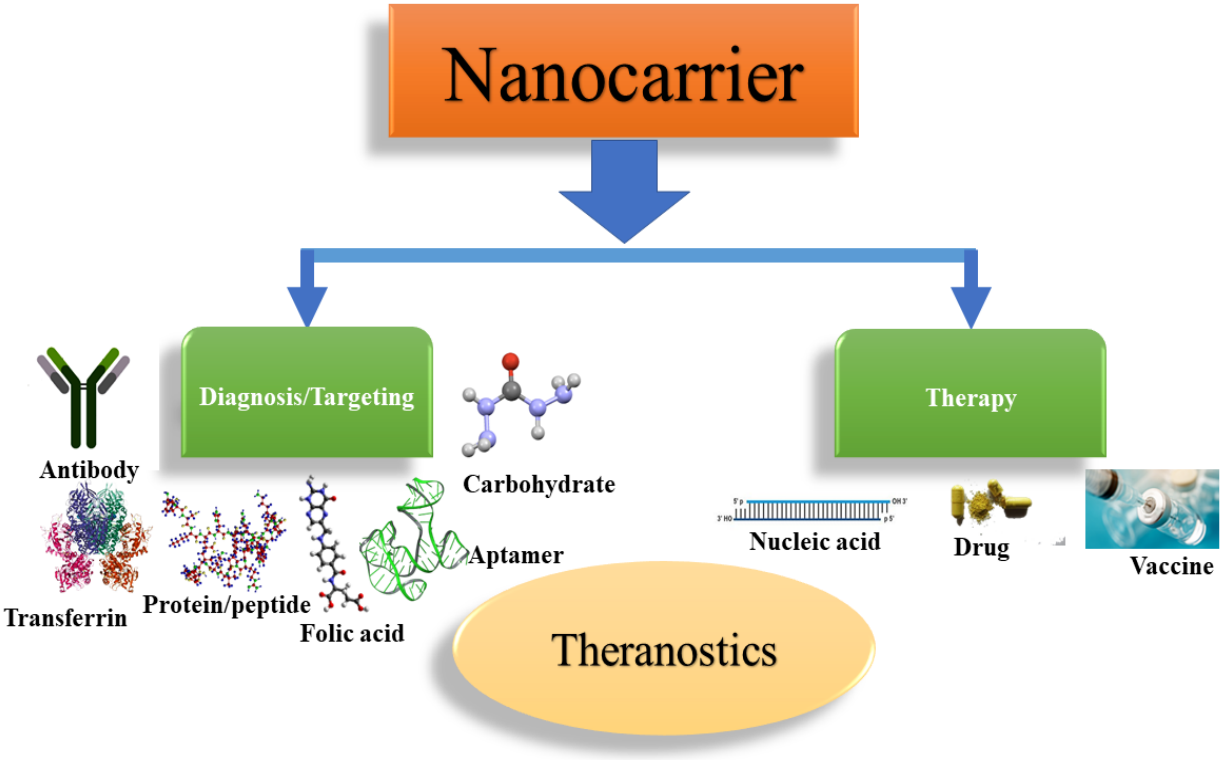

Figure 1.1. A pictorial representation of theranostics system including diagnosis/therapeutic agent along with nanocarrier.

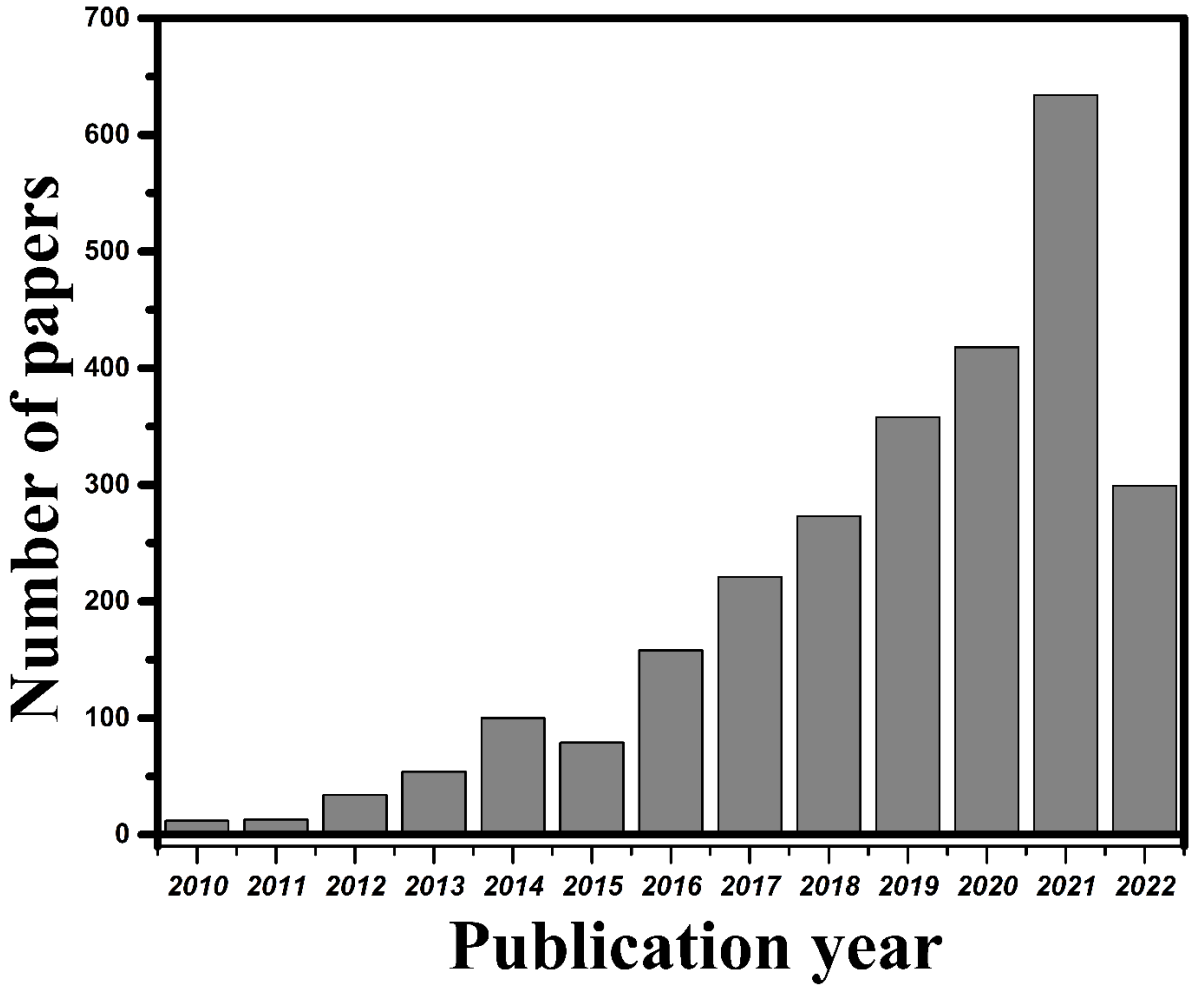

Figure 1.2. Bar-graph showing number of research papers published each year from 2010 to 2022 in the online database "Scopus'' searched using the keyword 'lipid nanocarriers and cancer theranostics.'

Various approaches have been utilized to deliver therapeutic medications to tumor sites, namely active targeting, passive targeting, and triggered drug delivery using nanocarriers **[12]**. The passive targeting is carried out through a perforated blood vessel system, allowing the nanocarriers to cross the membrane. Meanwhile, poorly formed lymphatic capillaries in tumor tissue limit backflow, resulting in the accumulation of nanocarriers in the tumor tissue **[13,14]**. On the other hand, the active targeting is facilitated by the active uptake of nanocarriers by receptor overexpressed tumor cells through their interactions with ligands**[15]**.

2. Types of NLBCs

Based on the construction processes and the physicochemical features of the formulations, NLBCs are divided into five classes: liposomes, niosomes, solid lipid nanoparticles (SLNs), nanostructured lipid carriers **(**NLCs), and lipid polymer hybrid nanoparticles (LPHNs).

2.1. Liposomes

Liposomes, the first-generation nanomedicines, were the first to undergo a successful transition from concept to commercialization. Since their discovery in the 1960s, various technological developments have been made to increase their effectiveness, ranging from liposome preparation to liposome targeting. A timeline of liposome/lipid-based nanomedicines advancement over the last 60 years has been illustrated in **Figure 1.3**. The major structural component of the liposome is a phospholipid which aligns in a spherical structure when mixed with an aqueous solution **[16] (Figure 1.4A)**. Another important component of liposomes i.e. cholesterol helps in providing stability to the liposomal structure and also enhances the solubility of drugs in the blood circulation system **[17]**. During the preparation of liposomes, cholesterol may form large vesicles of sizes ranging from 0.025-2.5 (μm) **[17]**. In **Table 1.1**, we have discussed the composition, shape/size, methods of preparation, advantages, and certain limitations associated with liposomes. However, the developed liposome drug carriers can be taken up by the cells either by passive or active targeting. In the case of passive targeting, the drug-loaded liposome is taken up via molecular diffusion through the cell membrane. On the other hand, active targeting is carried out by surface modification of the carriers with specific ligands **[18]**. Several studies have been published on liposomal drug formulation for various biological applications. In one of the studies, polyethylene glycol is conjugated successfully to the lipid monomer using β-glutamic acid as a cross-linker. The developed liposomal vesicle is

attributed to stability, bioavailability, and also enhanced toxicity to cancerous cells **[19]**. Likewise, the bioavailability of curcumin in the MCF-7 cells is enhanced significantly with RGD (Arg-Gly-Asp) modified liposomes **[20]**. Another group of researchers came up with an interesting concept where anticancer drugs have been targeted to the brain tumor utilizing GLUT receptor which maintains the energy requirement of the brain. For the formulation, glucose is conjugated with liposomal vesicles through polyethylene glycol to overcome the blood-brain barrier **[21]**. Wang *et al*. have successfully utilized the highly activated glycolysis pathway in cancerous cells. The aforementioned metabolic pathway helps in raising the temperature and reducing the pH of the cells. Temperature-sensitive liposomal vesicles for active targeting of anti-cancer drugs have been developed in response to this phenomenon **[22]**. Recently, Petrini *et al.* formulated two types of liposomal-based delivery systems using thermosensitive 1,2-dipalmitoyl-sn-glycero-3-phosphodiglycerol modified polyethylene glycol and thermosensitive 1,2-dipalmitoyl-sn-glycero-3-phosphodiglycerol in separate experiments. Cellular interactions of these developed systems were analyzed using fluorescence imaging in solid tumors. The thermosensitive 1,2-dipalmitoyl-sn-glycero-3-phosphodiglycerol showed more efficient liposome cell interactions which resulted in higher drug accumulation and cell toxicity comparatively **[23]**.Therefore, the potential of liposomes as delivery systems for enhanced drug accumulation in the cells must be carefully evaluated and can further be explored to develop nanomedicines for theranostics.

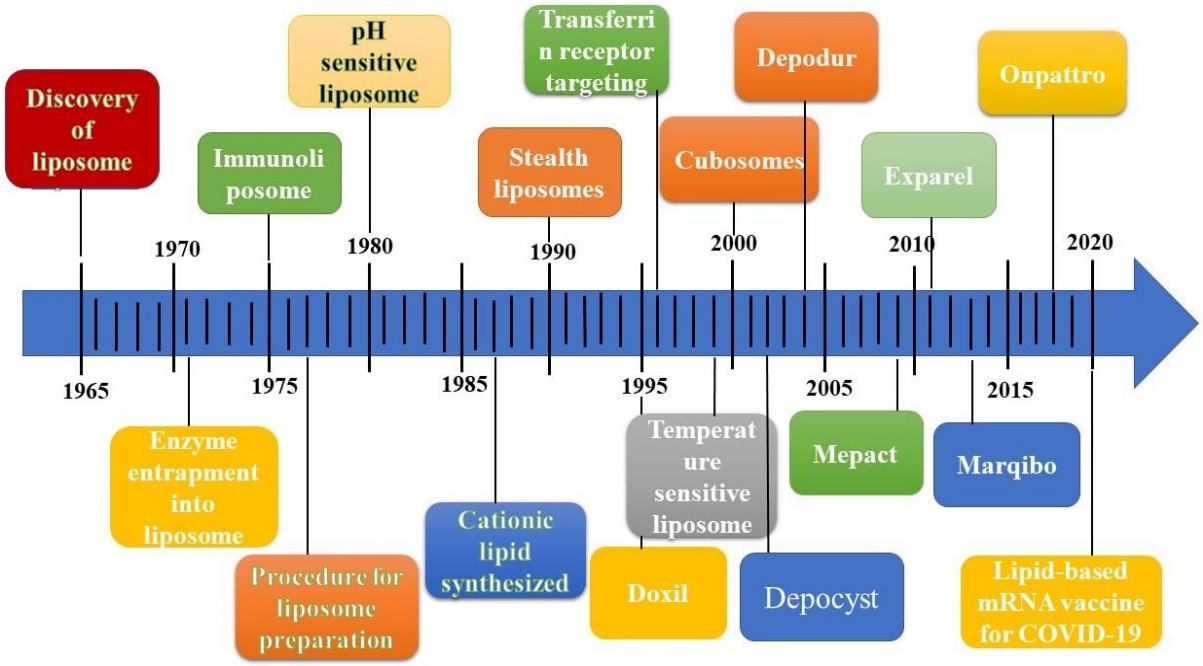

Figure 1.3. Timeline of liposome/lipid-based nanomedicine/nanocarrier advancement. The discovery of liposomes; Enzyme entrapment into liposomes; Immunoliposomes; Procedures for liposome formation; pH-sensitive liposomes; Cationic lipids synthesized; Stealth liposomes; Transferrin receptor targeting; Temperature-sensitive liposomes; Cubosomes. The earliest approved lipid-based nanomedicine, Doxil; The earliest FDA-approved lipid-based nucleic acid (siRNA) drug Onpattro; First LNP-based mRNA vaccines for COVID-19 approved. (CAS Content Collection).

2.2. Niosomes

Niosomes are being utilized as one of the potential carriers in drug transportation and are made up of non-ionic surfactants and amphipathic compounds **[24]**. Most likely, they resemble liposomes and are used for the transportation of amphiphilic drugs showing improved therapeutic efficacy **(Figure 1.4B)**. The physical parameters such as stability, membrane fluidity, and liquidity of the carriers are enhanced by varying the composition of the surfactants. Further, the incorporation of cholesterol and dicetyl-phosphate also helps in improving the stability of the niosomes **[25]**. Various properties such as biocompatibility, amenable for modification, the long half-life, and improved stability make niosomes a suitable drug delivery

carrier. Further, the major characteristics of niosomes including composition, size, methods of preparation, advantages, and limitations are presented in **Table 1.1**. In various studies, niosomes are used as one of the promising and leading drug delivery vehicles in the treatment of various diseases. Bartelds *et al*. came up with an alternative drug delivery technique and synthesized the niosomes using non-ionic surfactant and its physical properties were compared with liposomes. They found that the size of niosomes was significantly reduced and stabilized for a longer period of time as compared with liposome **[24]**. In another study, He *et al*. successfully synthesized the pegylated niosomes using a biocompatible surfactant, spans 60, and cholesterol for delivery of anti-cancerous drugs, paeonol. The developed niosomes have been improved significantly for the following features such as entrapment efficiency, drug uptake efficiency, *in-vitro* drug release kinetics, and also shelf-life of the drug **[26]**. Hence these studies show that the niosomes have huge potential to enhance the physical and pharmacokinetic properties of the active ingredients and also attract researchers to use them as drug delivery carriers.

2.3. Solid lipid nanoparticles (SLNs)

Numerous characteristic features including high surface to volume ratio, non-immunogenicity, biodegradability, and solid nature at room temperature with an easy scale-up process make SLNs a suitable drug delivery carrier **[27]**. However, SLNs have certain limitations, including limited drug loading efficiency and drug discharge during the crystallization process **[28]**. In the preparation of the SLNs, oil is replaced by solid lipids in the emulsion, as illustrated in **Figure 1.4C.** Moreover, highly purified lipid monomers along with various features of SLNs are well illustrated and explained in **Table1.1.** Various studies have been reported so far for SLNs-based formulations that are used in disease theranostics. In one of the studies, SLNs-based drug delivery systems have been constructed for STAT 3 inhibitors against triple-negative breast cancer cells. Further, the Box-Behnken design has been used for the

optimization of the shape, size, and charge of the developed SLNs **[29]**. In another study, a unique magnetic-based SLNs system has been designed for the delivery of the anticancer drug, paclitaxel via diffusion technique. The experimental results showed that the melting point of the lipids decreased upon coating the magnetic particles onto lipid monomers. Further, a reduction in the melting point of the particles provides stability and controls the drug release pattern in a concentration-dependent manner. In a fascinating study by Wang *et al*., SLNs were constructed using 2^2 factorial designs for the transportation of two monoterpenes (citral and geraniol). Anti-inflammatory activity of the citral and geraniol was initially tested against WAW 264.7 cell line. Further, the formulated SLNs were evaluated for long-term stability and the obtained data showed that they were steady for a month **[30]**. Similarly, SLNs were constructed using structural components, cetyl palmitate, and polysorbate 80 through the high-pressure homogenization method. In the construction, indirubin was used as a potential anti-cancer drug for the treatment of the glioblastoma multiforme (GBM). Further, indirubin-coupled SLNs showed higher anti-tumor activity than that of free indirubin against GBM cell lines **[31]**. Zho *et al.* conducted an interesting study where absorption of tilmicosin in the duodenum was enhanced 2.72-fold. They constructed SLNs using enteric granule containing P-gp inhibitor which reduces the efflux mechanism of the tilmicosin **[32]**. The above experimental findings suggest that the SLNs can be engineered in the near future to meet the specific needs of a drug delivery carrier.

Table 1.1: List of various types of lipid-based nanocarriers.

Lipid nanocarriers	**Composition**	**Shape/Size**	**Methods of preparation**	**Advantages**	**Disadvantages**	**References**
Liposomes	Phosphatidylcholine, Phosphatidylserine, 1,2-dipalmitoyl Sn-glycero-phosphocholine monohydrate	Spherical/ ≤ 1 µm	Thin-film hydration/ Solvent dispersion method/Detergent method	Facilitates controlled release of drugs	Costly scale-up process, Instability in long time storage	**[125,126,127,128 129,130]**
Niosomes	Alkyl ethers/Alkyl amide/Fatty alcohols/Fatty acid/ Cholesterol	Spherical/ ≤ 1 µm	Thin-film hydration/Sonication/Microfluidization/Reverse phase evaporation method	Sustained release of drugs, osmotically stable, non-immunogenic	High production cost, reduced shelf-life, time-consuming process	**[131,132]**
Solid lipid nanoparticles	Phospholipid (90 NG)/Glycerol tristearate/Glycerol	Spherical/50-1000 µm	Sonication/Double emulsion method/Spray drying/Solvent emulsification	Stable over time, Hydrophilic & lipophilic drugs both can be encapsulated, enhanced	Drugs dissolved in the lipid matrix, low drugs encapsulation efficiency	**[125,32,33,34, 26]**

	monostearate/Cetyl palmitate			bioavailability, easy scale-up process		
Nanostructured lipid carriers	Tristearin/Stearic acid/Cetyl palmitate/Cholesterol/ Precirol ATO5/Compritol^{R}888/ Dynasan^{R}116/Softisan ^{R}154/Imwitor^{R}900P/G elot^{R}64/Emulcire 61/Triglycerides/Paraf fin oil/Oleic acid/Squalene/Isoprop yl myristate/Vitamin E	Several nm to 3 µm	homogenization/Hot homogenization/Microem ulsion	Increased solubility, enhanced storage capacity, increased half-life, improved bioavailability, improved permeability	High operating temperatures required, not stable over a long time	**[3538]**
Lipid polymer hybrid nanoparticles	Polymer-PLGA/PCL/Poly-L-arginine/PEI/DSPE/PL A/Poly-L-lactide/DSPE Lipid-Lecithin/DSPE/DLPC/ DMPE/Cholesterol	Spherical/6 5-279.9 nm	Nanoprecipitation/Emulsif ication-solvent evaporation	High stability, long half-life, appropriate particle size, amenability for surface modification, sustained release of drugs	Expensive, tedious scale-up process, clumsy lab-scale preparation, dilution rate increased	**[30,119]**

2.4. Nanostructured lipid carriers (NLCs)

NLCs, called second-generation NLBCs are synthesized using two different types of monomers **[39]** which are both solid and liquid in nature. This kind of carrier is capable of overcoming problems associated with the most common type of carriers such as low drug loading efficiency, and drug leakage during crystallization **(Figure 1.4D)**. Numerous properties such as reduced *in vivo* toxicity, the capability of targeted delivery, and amenability for surface modification make NLCs a prominent drug delivery vehicle **[40]**. The addition of solid lipid into liquid-lipid turns the matrix into a more crystal disorder structure and this facilitates the high entrapment of the drugs **[41]**. The main characteristics of NLCs including composition, size, methods of preparation, advantages, and limitations are presented in **Table 1.1**. Many researchers have successfully synthesized NLCs using various methods and the constructed formulations have been used for biological applications. In a study, Fluvastatin-alpha lipoic-ellagic acid was successfully conjugated with NLCs (FLV-ALA-EA-NLCs) and the developed FLV-ALA-EA-NLCs were studied *in vivo* system. The findings revealed that it can trigger more pre-G1 phase in prostate cancer patients than the free form of FLV-ALA-EA **[42]**. In a similar example, the bioavailability of thymoquinone is enhanced by using NLCs against liver cancer cells **[43]**. Also, noteworthy, *in vitro* cell toxicity of the orcinol glucoside loaded polyethylene glycol-25/55-stearate modified NLCs was tested against hepatoma cell line **[44]**. In another work, NLCs were synthesized using three different structural components, glyceryl monostearate (solid -lipid), decanoyl/octanoyl-glycerides (liquid-lipid), and tween 80 (surfactant). The oral administration of the above-formulated 6-gingerol-NLCs showed enhanced bio-availability when compared with the free form of 6-gingerol in a mouse model **[45]**. In an interesting investigation, NLCs were optimized using Precirol^{R} ATO 5(solid -lipid) and Transcutol^{R}HP (liquid-lipid) along with various combinations of the surfactants. The best combination was selected for the delivery of hydrochlorothiazide to enhance oral

bioavailability. Moreover, data showed that the optimized combination improved the pharmaceutical and physical properties such as particle size, drug loading efficiency, drug release rate, solubility, and stability of NLCs as well **[46]**. Similarly, a group of researchers investigated the stability of lidocaine-loaded NLCs at different temperatures using various modeling methods **[47]**. The aforementioned examples depicted that the surface of the NLCs can be modified with various types of ligands for the targeted delivery of active ingredients. Also, the physical and pharmacokinetic properties of the carrier can be improved by optimizing the composition of lipid monomers.

2.5. Lipid polymer hybrid nanoparticles (LPHNs)

LPHNs are the next-generation based nanocarriers that merge the characteristics of lipid and polymer. Several limitations are associated with lipids when used alone as a drug delivery system. On a contrary, polymeric nanoparticles have emerged as a potential carrier due to their small size, long-term stability, high drug loading efficiency, and controlled drug release with an improved half-life of the drugs while circulating in the reticuloendothelial system **[48]**. Hence, the researchers are working to develop a unique system comprising two different nanocarriers. Such a dihybrid system shows an effective result that can be achieved due to its synchronous effects. Further, LPHNs consist of three layers; (i) the core layer containing polymeric matrix, (ii) the middle layer comprising the lipid layer, and (iii) the outer pegylated layer, facilitating the systemic circulation by avoiding the immune system **(Figure 1.4E).** In the developed system, the middle layer provides stability to the inner layer by preventing water entry **[49]**. The exact mechanism of synthesis of lipid-polymer hybrid integration is still unclear. However, in a one-step preparation of LPHNs, the preformed polymeric solution facilitates the precipitation of nanoparticles and aggregation of lipid monomer around the polymeric matrix. Whereas, in the case of the two-step process, lipid suspension is synthesized through the lipid thin film hydration technique and is subsequently precipitated in an organic

solution comprising polymeric nanoparticles **[50]**. Further, the main characteristics of LPHNs including composition, size, methods of preparation, advantages, and limitations are presented in **Table 1.1.** Several studies based on LPHNs were successfully undertaken by researchers for precise and controlled drug delivery. In one such study, a stable hybrid system was successfully developed using 1,2-dilauroyl-sn-glycero-3-phosphocholine and poly lactic co-glycosidic acid as structural components. In this case, the monolayer of the lipid surrounds the polymeric matrix containing docetaxel, a potential anticancer drug, and folic acid is conjugated to the surface of the lipid-polymer carrier. The constructed carrier is attributed to increased encapsulation efficiency, reduced size, better bioavailability, and also stability for a longer period **[51]**. Similarly in another study, doxorubicin retention and its accumulation in P-glycoprotein expressed cancer cell line is enhanced using LPHNs system. In this investigation, an endocytosis inhibition assay was also performed to understand the mechanism of cellular uptake of the developed system. Moreover, images obtained from fluorescence microscopy confirmed that the cellular uptake of the doxorubicin gets enhanced significantly through the formulated nanocarriers **[52]**. Bochicchio *et al.* came up with recent technology to develop the hybrid nano system using lipid and polymer for overcoming production limitations such as high energy requirements, poor productivity, and extensive set-up required at the industrial level. Further, the developed LPHNs system has been utilized for various applications such as breast cancer therapy, liver cancer therapy, and also vaccine development for SARS-CoV-2 **[53]**. Hong *et al*. developed a curcumin-loaded LPHNs system using enzyme-directed peptides and polycyclic lipid to achieve site targeted delivery against U 251 cell line and mouse tumor tissue. They suggested that the formulated hybrid constructs facilitate better drug accumulation in the U 251 cells, and prevent the growth of the tumor tissues with low toxic effects **[54]**. In a most recent study, Qin *et al*. constructed an LPHNs system to enhance the oral bioavailability of BCS 1V drug by 8-fold since it gets rapidly metabolized by P450 enzymes. In this hybrid

system, they incorporated P-gp inhibitors, CYP 450 inhibitors, and paclitaxel, a potential anticancer drug **[55]**. All the above examples revealed that the LPHNs system show enhanced stability, crystallinity, encapsulation efficiency, and drug delivery in a controlled manner in the biological system.

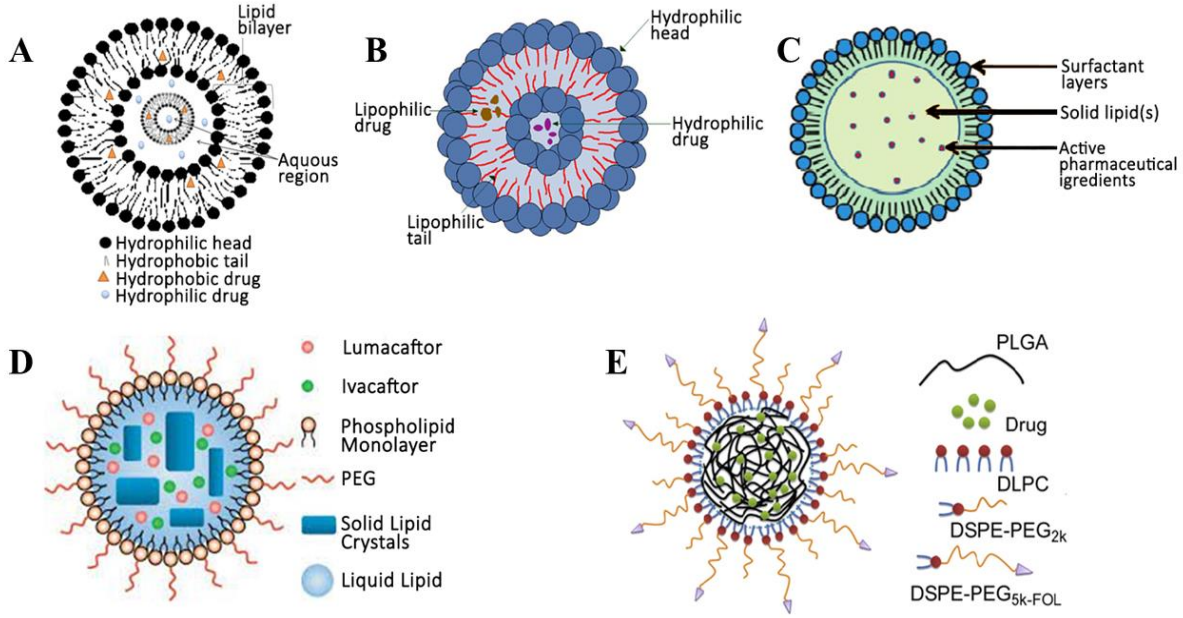

Figure 1.4. Schematic representation of five classes of lipid-based nanocarriers (A) Liposomes (Reproduced with permission from Soni *et al.*) **[56]** (B) Niosomes (Reproduced with permission from khoee *et al.*) **[57]**(C) Solid lipid nanoparticles (SLN) (Reproduced with permission from Tekade *et al.*) **[58]**. (D) Nanostructured lipid carriers (NLCs) (Reproduced with permission from Garbuzenko *et al.*) **[59]** (E) Lipid polymer hybrid nanoparticles (LPHNs) (Reproduced with permission from Liu *et al.*) **[51]**.

3. Preparation methods of NLBCs

3.1. Double emulsion method

A double emulsion method is an effective approach for encapsulating hydrophilic active pharmaceuticals within lipid carriers **[60]**. Primarily, hydrophilic drugs are dissolved in an aqueous solvent, subsequently, the formed solution is added to the melted lipid solution to form a single emulsion. The formed single emulsion gets stabilized by surfactant comprising a

secondary aqueous solution. Further, this single emulsion is mixed with the aqueous solution of the hydrophilic stabilizer to form a double emulsion **(Figure 1.5A).** Consequently, the formed double emulsion is purified using centrifugation or filtration techniques in cold conditions. Since this method utilizes a hydrophilic surfactant, the surface modification can be easily carried out. However, the particle size obtained through this method is relatively larger and requires further processing.

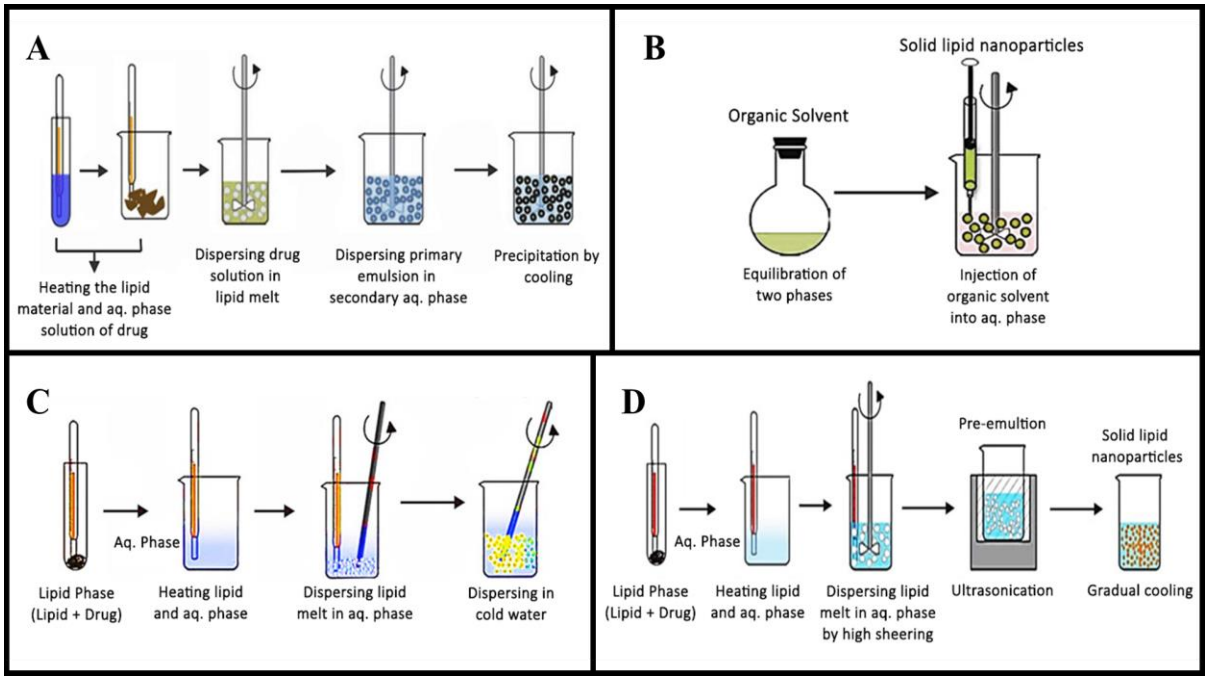

Figure 1.5. Schematic illustration of various techniques for synthesis of nano lipid-based carriers (A) Double emulsion method (B)Solvent injection method (C) Microemulsion method (D) Ultrasonication method. (Reproduced with permission from Ganesan *et al*.**[61]**

3.2. Solvent injection method

This method is based on the diffusion principle and employs simple methods to produce lipid nanoparticles. First, the lipid is dissolved in water-miscible solvents such as methanol, acetone, and isopropanol to obtain the organic phase. Subsequently, the organic phase is injected into a surfactant-containing aqueous solution through a syringe needle **(Figure 1.5B) [62]**. The following two factors influence lipid nanoparticles synthesis:

1. Progressive solvent removal from lipid droplets into the aqueous phase reduces droplet size while increasing the lipid concentration at the same time.

2. When the solvent is removed from the lipid droplets, the local interfacial tension at the droplets' surface increases, causing the droplets to shrink even further. The size of the particles can also be affected by changes in process parameters such as lipid concentration in the organic solvent, aqueous phase viscosity, and solvent volume.

3.3. Microemulsion method

This approach has been employed to produce lipid nanoparticles since the early 1990s due to the minimum requirement of energy. Owing to the high lipid/surfactant ratio, lipid nanoparticles are formed spontaneously using this approach. In the first step, the lipid is melted above the transition temperature, then subsequently mixed with a pre-heated aqueous solution and stirred gently to obtain microemulsion. While in the second step, the emulsion is poured into a high volume of cold water under reasonable stirring conditions to solidify the droplets **(Figure 1.5C).** The obtained nanoparticles have a spherical shape and their size is in the nanometer (nm) range. However, the major problem of this method is that as the process needs a highly diluted solution, it requires evaporation of a liquid to obtain a more concentrated form of the particles. Moreover, a high ratio of lipid/surfactant is required in this technique which raises the production cost of the lipid nanoparticles **[63]**.

3.4. Ultrasonication method

This method is based on a dispersion technique that utilizes high energy to obtain the lipid nanoparticles**.** Initially, the lipid is melted above the transition temperature, and then the melted solution is added gradually to the pre-warm aqueous solution to obtain the emulsion under a magnetic stirrer. In the next step, large droplets of the emulsion are reduced using ultrasonication at a particular amplitude under ice-containing vessels. Further, the emulsion is gradually cooled and it leads to the formation of lipid nanoparticles which are then subsequently purified using ultracentrifugation **(Figure 1.5D) [64]**.

3.5. Spray-drying method

In this approach, the lipid is dissolved in the same solvent as the active component to make a solution. After that, the solution is further passed through the atomizer of the spray drying chamber and dried through hot drying gas **(Figure 1.6A) [65]**. In this system, nitrogen gas is provided to maintain the inert environment since the organic solvent is used in the process. Further, the liquid spray is introduced in the drying chamber using various nozzles including hydraulic, ultrasonic, rotary, and fluid nozzles **[66]**. Once spray dispersion is in contact with hot drying gas, the solvent starts evaporating leaving the sample dry. In the next step, particles are removed from the gas stream through a cyclone separator which is decorated within the system. However, a spray dryer is operated in recycling mode in many industries to obtain lipid nanoparticles on a massive scale. Solvent-loaded drying gas is passed through the condenser, re-warmed, and returned to the drying chamber in this system**.** The operating parameters for the spray drying in the closed loop vary from those employed in single pass mode.

3.6. Emulsification-solvent evaporation method

This method comprises three steps for the production of lipid nanoparticles 1. Preparation of organic phase, 2. Pre-emulsification, and 3. Nano-emulsification **[67]**. In the first step, lipid

along with lipophilic drug is dissolved in an organic solvent followed by dispersion through magnetic stirring. Further, the formed organic phase is gradually added to the aqueous phase under a high-speed homogenizer to obtain droplets of the particles in the second step. While in the third step, the formed droplets of the particle are reduced to the nanometer range through a high-pressure homogenizer/sonicator **(Figure 1.6B).** Later, the dispersion of the nano-sized particles is kept on a magnetic stirrer overnight to evaporate the solvent under reduced pressure. The approach described above can be used to make nanoparticles with improved physiochemical, and pharmacokinetic properties **[68]**.

3.7. Supercritical fluid method

Column based extraction method is also known as the supercritical fluid extraction method **[69]**.In this method, the organic phase is prepared by dissolving the lipid, lipophilic drugs, and surfactants in an organic solvent such as; chloroform to form a solution. Subsequently, the solution is mixed with an aqueous solution containing a co-surfactant. Further, the blend is getting through a high-speed homogenizer to make the emulsion. The formed emulsion is passed through one end of the column at a fixed flow rate and supercritical fluid is introduced through the other ends of the column to counter the flow rate at constant temperature **(Figure 1.6C).** The purified lipid nanoparticles are extracted by periodic removal of solvent from the emulsion.

3.8. Hot homogenization method

In this method, lipids along with active pharmaceuticals are dissolved in an organic solvent. After that, the organic solution containing lipids is melted and mixed with an aqueous surfactant having the same temperature. Dispersing lipid melt in an aqueous phase using the shear device results in the formation of a pre-emulsion. Subsequently, pre-emulsion was then ultrasonicated and in the next step, the hot homogenizer equipped with a piston gap is then

used to amalgamate the pre-heated solution into the colloidal solution. Further, the homogenized solution was allowed to cool at ambient temperature to obtain the liquid droplet crystallinity **(Figure 1.6D) [70]**. It is reported that the refrigeration condition and sub-zero temperatures are required to achieve the appropriate size of the particles **[71,72]**. The pre-heated solution significantly affects the particle size, and degradability of drugs therefore homogenization cycle needs to be repeated several times.

3.9. Cold homogenization method

This method is being utilized to solve challenges related to the hot homogenization process, such as drug degradability and crystallization difficulties of nano-emulsions **[72]**. The lipid is heated above the transition temperature in this approach, and then drugs are introduced to the molten stage of the lipids. Further, molten lipids along with drugs are allowed to solidify in a rapid cooling method using liquid nitrogen **[68]**. In the process of rapid cooling, drugs are equally distributed within lipid carriers. After that, the solid particles of the lipids are converted into fine particles or microparticles using milling. In the next step, microparticles are dispersed into a surfactant-containing aqueous solution and finally, the dispersed solution is passed through the homogenizer to produce the lipid nanoparticles **(Figure 1.6D)**.

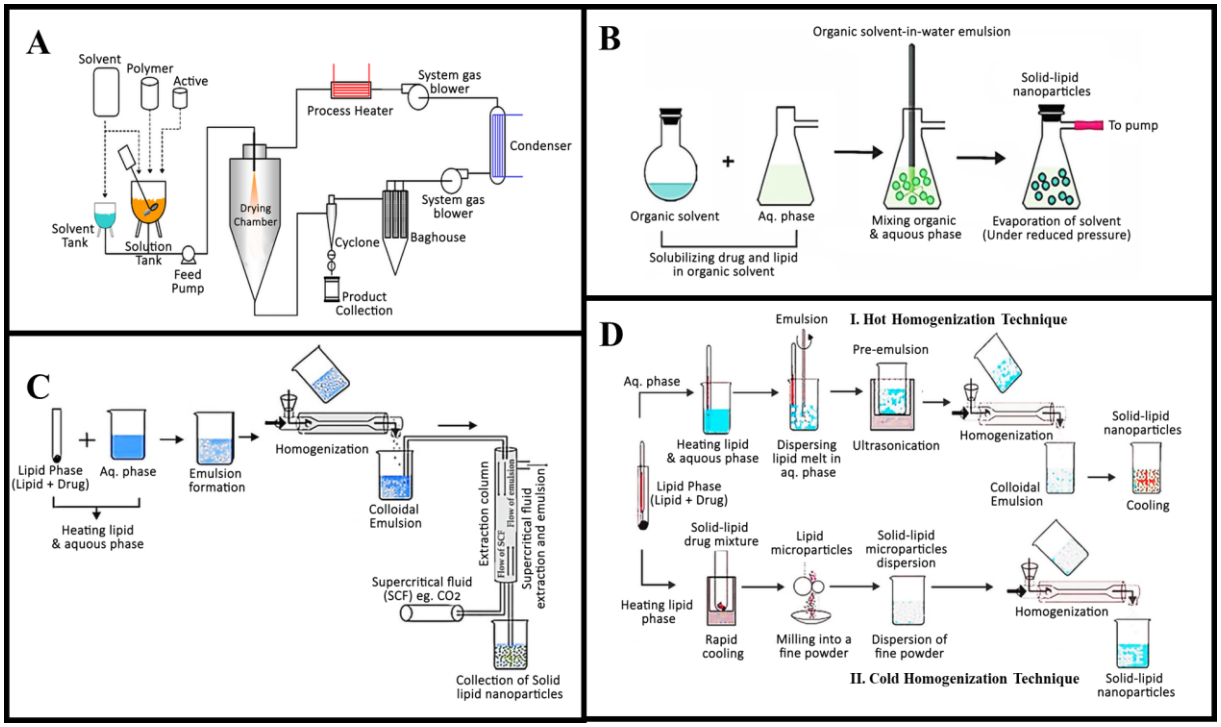

Figure 1.6. Schematic illustration of various techniques for synthesis of nano lipid-based carriers (A) Spray drying method (B)Emulsification- solvent evaporation method (C) Supercritical fluid method (D) Hot/Cold homogenization method. (Reproduced with permission from Ganesan *et al*.,2017) **[61]**.

4. Stability and Drawbacks

In recent years, nanocarrier technology has seen remarkable progress in the effective delivery of therapeutic drugs **[73]**. Lipids have been widely explored as drug delivery carriers for boosting the bioavailability of drugs, either by increasing the dissolution rate or by improving their water solubility **[74]**. NLBCs, which are made up of natural or synthetic lipids, are a versatile nanoplatform for developing improved drug delivery systems **[73]**. Despite tremendous advancement in the development of such dynamic drug delivery systems, one of the most important constraints is their poor colloidal stability, which arises from interactions with the complex environment in the biological fluids **[73]**. For instance, the shelf life of various nano lipid-based formulations is affected by physical interactions, including aggregation and fusion **[69,70]**. Similarly, the functioning of phospholipid bilayers can be

influenced by the chemical interactions of nanocarriers with biological fluids. Peroxidation of lipids and hydrolysis of the ester bonds, which connect the glycerol backbone and fatty acid chain are the two major types of chemical reactions which affect stability **[75,76]**. Moreover, NLBCs formulation stability or shelf life is also determined by the types and nature of lipid monomers utilized in the system. Unsaturated lipids, in general, are more susceptible to oxidation and have a lower shelf life **[75]**. Long-term storage of NLBCs can also cause stability problems such as sedimentation and agglomeration **[74]**. Pharmaceutical lipid-based nanomedicine is frequently subjected to stability testing in various climatic conditions **[74,76]**. It is required during the development of a product to determine its shelf life and appropriate storage conditions **[76]**. Nano lipid-based formulations remain stable at low temperatures (4ºC) for 3 months and 6 months of storage period. It is observed that the particle size, encapsulation efficiency, polydispersity index (PDI), and zeta potential are affected at the storage condition (25 ºC/ 60% RH). The value for particle size and PDI are significantly increased however, the zeta potential value decreases with the degradation of the particles at the above storage conditions **[77]**. Increasing the particle size has been exploited by some researchers to determine poor solid lipid nanoparticle stability **[74]**. It can be interpreted that time, temperature and humidity influence the overall stability of the system. Also, the PDI value was increased due to the formation of agglomerates in the nanosystem **[77]**. Furthermore, nanosystem deterioration occurs at high temperatures due to higher kinetic energy**[74]**. The most effective strategy to improve NLBCs stability is to develop a suitable formulation, which necessitates choosing the right lipid composition and concentration to extend its shelf life **[78]**. Cholesterol and its derivatives, for example, can reduce the lipid bilayer permeability **[78]**. Physical stabilization can be achieved by ionic surfactants, steric stabilizers, or by the inclusion of lipid nanoparticles into creams or hydrogels. Additionally, by modifying the size, crystallinity, and zeta potential of NLBCs, we can improve physical stability. Crystallization

can also cause a variety of physical instabilities; thus, it is important to characterize the lipids using X-ray diffraction (XRD) and Nuclear magnetic resonance (NMR). Electrostatically stabilized systems have zeta potentials higher than 30 mV. Antioxidants namely α-tocopherol, butyl hydroxyl anisole (BHA), and butyl hydroxyl toluene (BHT) can help to maintain the chemical stability of NLBCs against oxidation. Likewise removing water either by freeze-drying or spray drying might increase the chemical stability of the formulated system **[74]**.

5. Lyophilization (freeze-drying)

Freeze-drying also called lyophilization, is an industrial process that involves the sublimation and desorption process to remove water from a frozen sample using a vacuum system **[74,79]**. This drying procedure is used to turn labile material solutions into solids that are stable enough to be distributed and stored **[78]**. Freeze-drying is the most popular method for prolonging the shelf life of thermosensitive medicines contained within NLBCs **[78]**. Cryoprotectants namely, sucrose, hydroxypropyl-β-cyclodextrin, and trehalose further enhance the stability of the carriers during the freeze-drying process **[78,79]**. Hence, stability is an important factor in ensuring drug safety and efficacy. Physical and chemical stability is one of the major drawbacks associated with lipid nanocarrier system in terms of solubility, agglomerations, precipitation, hydrolysis, oxidation, leakage of drugs, and also prone to chemical reactions with other compounds. Various formulative approaches such as the addition of an antioxidant, use of highly charged particles, a lipid with a high transition phase, saturated lipid, and storage at a low temperature are not as effective to overcome the mentioned drawbacks associated with them. One alternative is to maintain the stability of the system by drying the nanocarriers using a lyophilizer because all the aforementioned phenomena happen in the aqueous phase. Whenever lipid nanomedicine is administered orally/pulmonary routes, the drying of the formulations becomes useful **[80]**.On the other hand, freezing and drying cause irreversibility or structural alterations to the NLBCs system since lipid monomer assembles to various forms

of the structure in the presence of water **[81]**. Primarily, freeze-drying includes three steps freezing, primary drying, and secondary drying which hampered the membrane integrity. At above the freezing temperature, the lipid-water suspension is separated into two phases viz. bulk phase and lamellar phase. The water molecule in the bulk phase starts ice crystal formation leading to closeness among lipid carriers. Simultaneously, in the lamellar phase, ice crystal formation triggers the reduction in the spacing of phospholipid head groups resulting in the lateral expansion of the membrane **[82]**. On the other hand, slow freezing reduces the osmotic shock and supercooling leading to fine ice crystal formation in the inner layer and subsequently preventing drug leakage. It is hypothesized that slow freezing facilitates the lipid carriers to reform their structure damaged due to osmotic pressure/ mechanical forces. Further, drying facilitates the dehydration of lipid bilayer resulting in the hydrophobic acyl chains interaction. Such interactions increase the packaging density of lipid bilayer resulting in the hexagonal structure of lipid shifting to ribbon phase structure in which the bilayer is packed to form 2-dimensional lattice **(Figure 1.7A).** The increased ordering of the lipid phase subsequently decreases the tilt angle of the hydrocarbon backbone. The above phenomenon increases the transition temperature (Tm) of the lipid bilayer. However, Tm also depends on other factors such as the polar nature of the head group, length of acyl chain, degree of saturation, and lipid affinity with surrounding water molecules. Tm is one of the important factors which influences the stability of NLBCs **[83]**. However, the Tm can be controlled using suitable excipient such as sugar and disaccharide which interacts with the polar head group of the lipid resulting structural loss due to drying is minimized **(Figure 1.7B).** The cholesterol also decreases the Tm by increasing the hydrocarbon tilt angle which further stabilizes the NLBCs. Furthermore, due to the presence of the OH group on the interfacial region, it may interact with the polar heads of lipids via H-bond formation **[84]**. A group the researchers also investigated that the presence of cholesterol, the leaching of drugs from the core of the nanocarrier can be controlled

[85].Primary drying is one of the key process parameters which can be inspected as a balance between mass and heat transfer in the system. Since the rate of mass transfer from solid is directly proportional to the latent heat of sublimation. Therefore, allowable heat required for sublimation is considered in terms of glass transition temperature (Tg). At this point, the maximally frozen concentrated solution is in a glassy state with a viscosity range of $10^{13\text{-}14}$P. On the other hand, the mobility of the system increases and the system moves from solid to liquid state at the temperature above the Tg **[86]**. Furthermore, Tg is also considered for the prediction of collapse temperature (T_C) which does not support the structure of NLBCs. At the above Tg, the system can be collapsed or aggregated with concomitant vesicle fusion. Secondary drying determines the residual moisture contents in the product. The moisture content is actually plasticizing water embedded in a glassy matrix which influences the long-term stability of the carriers. The amount of water that cannot be eliminated by sublimation is the most important component that determines the operative parameters of this stage **[87]**. Therefore, freeze-drying process should be monitored to take care of lipid nanocarrier formulation using freeze-drying microscopy and time-domain optical coherence tomography.

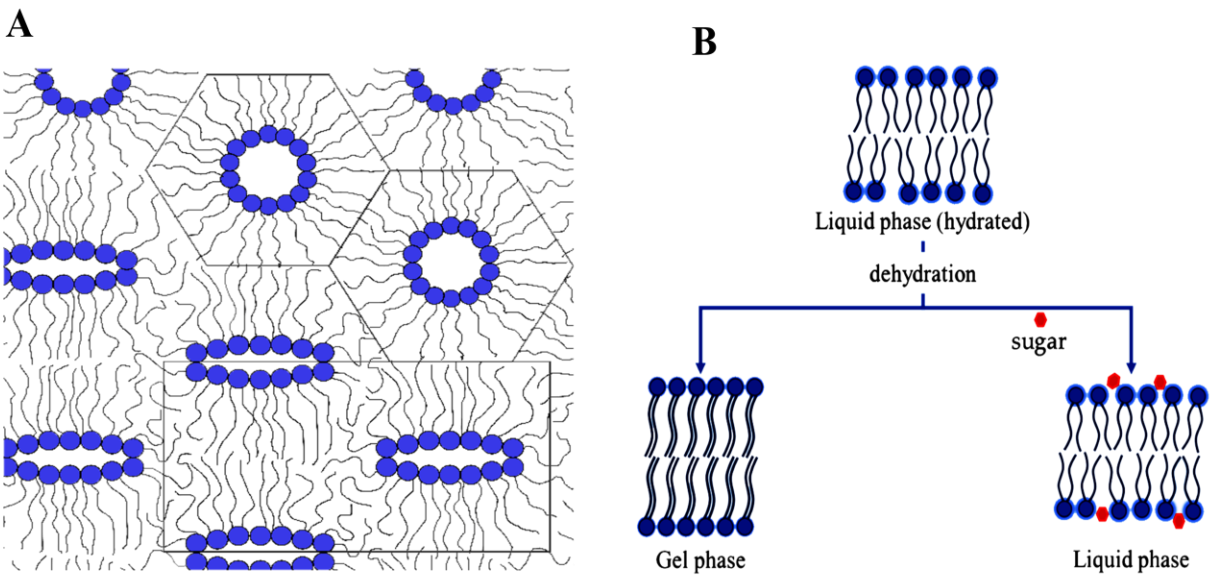

Figure 1.7. (A)The lipid bilayer exists in two phases. The lipid head group surrounds the water channel before drying (Hexagonal phase). The lipid bilayer is packed into a two-dimensional lattice after drying (Ribbon phase). Reproduced with permission from Kent *et al*. **[88]**. (B) Orientation of lipid bilayer before and after drying in the presence and absence of sugars. Reproduced with permission from Franze *et al*.**[83]**.

6. Targeting moieties

The primary goal to utilize surface-expressed receptors is to successfully deliver drugs and detect the malignancies using ligand decorated NLBCs. Nanocarrier design and formulation laid the basis for receptor-mediated cellular entrance via endocytosis **[89]**. The proper selection of ligands is crucial in developing the potent carriers to selectively diagnose following preferential uptake of drugs in the cancerous cells **[90]**. A list of ligands and their target molecules with nanocarriers is illustrated in **Table 1.2**.

Table 2.2: List of ligands and their target molecules with nanocarriers.

S. No.	Ligand	Targets	Nanocarriers	Tumors	References
1	RGDF peptides	Integrin receptors ($\alpha v\beta_3$, $\alpha v\beta_5$)	Micelles	Murine hepatic cacinoma (H22 cell)	**[91]**
2	A54 peptide	Hepatocarcinoma cell	NLCs	Hepatic tumor (Bel-7402 cell)	**[92]**
3	cRGD	Integrin receptors ($\alpha v\beta_3$)	Micelles	Astrocytoma (U87 cells)	**[93]**
4	cRGD	Integrin receptors ($\alpha v\beta_3$)	Micelles	Brain tumor (U87MG cell)	**[94]**
5	Lyp-1 peptide	Tumor site	Micelles	Breast cancer (4T1 cell)	**[95]**
6	hEGF ligands	EGFR	Liposome-like nanovesicles	Ductal carcinoma of breast (BT474 cell)	**[96]**
7	Hexapeptide ligand AE	EGFR	Micelles	Astrocytoma (U87 cell)	**[97]**
8	Anti-CD44 antibody	EGFR	Micelles	Breast tumor (MCF7 cell)	**[98]**
9	HIV trans-activating	Nuclear pore complexes	Micelles	Cervical cancer (HeLa cell)	**[99]**

	transcriptional activator (TAT) peptide				
10	EGa1 nanobodies	EGFR	Micelles	Squamous cell carcinoma (14C cell)	**[100]**
11	Hyaluronic Acid	CD 44	SLNs	Melanoma cancer (B16F10 cell)	**[101]**
12	Galactose	lectin receptors	SLNs	Adenocarcinoma (A549 cells)	**[102]**
13	transferrin	Tf receptors	NLCs	Human non-small cell lung carcinoma cell line (NCl-H460 cells)	**[103]**
14	Folate	folate receptor	Liposome-PLGA Nanoparticles	epidermal carcinoma cell line, K.B. cells, lung carcinoma cell line, A549 cells	**[104]**

6.1 Transferrin receptor

A glycoprotein helps in iron capturing from the circulatory system and facilitates cellular internalization through receptor-mediated endocytosis. Also, it involves controlling the iron influx in cells, thereby sustaining homeostasis. The receptor is a prospective target site for specific ligands and antibodies because of elevated levels of its expression, which can be 100 fold greater than the normal cell expression **[105]**. The fundamentals of chemistry play a key role in conjugating specific ligands or antibodies with the surface of NLBC for precise, controlled, and site-directed delivery of anti-cancer drugs with selective detection. Aqueous stability testing in a biologically simulated environment reveals that the transferrin-conjugated NLBCs (Tf-NLBCs) are more stable and facilitate drug release in a targeted manner. **Figure 1.8A** shows the step-by-step synthesis of transferrin conjugated NLBCs, and the mouse xenograft model determines the biodistribution of the formulation. The structural components of lipid carriers are soya lecithin, oleic acid, tween 80, glyceryl monostearate, and PEG-PE, which provide strong stability and high encapsulation efficiency. The fate of the structure of the NLBCs is dependent on the types of lipid monomer used during the preparation of the carrier. The PEG-PE provides the PEG length and terminal moieties, making the covalent bond with the targeted Tf receptor. It was observed that the efficacy of Tf decorated NLBCs enhanced 10-fold as compared to free drugs. In the context of anti-cancer drug delivery to specific sites, numerous researchers constructed and evaluated the efficacy of Tf- NLBCs in the diverse cancer cell line and mouse models. In another study, Li *et al*. (2009) successfully utilized the overexpressed Tf receptor on the malignant cell surface for designing the nanocarrier. They constructed Tf-coupled liposomal vesicles for targeting doxorubicin as a potent anti-cancer drug. Film dispersion and ammonium sulfate gradient methods were employed to synthesize liposomal vesicles. Tf was functionalized on the cell surface of the

vesicles comprising DSPE-PEG_{2000}-COOH through EDC-NHS crosslinking. The developed construct was evaluated against cancerous cells, and it was found that the Tf-coupled liposomal vesicles accumulate more drugs inside the cells than uncoupled vesicles. However, the cytotoxic effect induced by the developed construct on normal cells was minimal. In addition, pharmacokinetics studies in tumor mice revealed that coupled vesicles effectively delivered the drugs to the targeted tissues; however, the concentration of the drugs was found to be minimum in non-targeted tissue such as the heart and kidney. The weight of the Tf conjugated doxorubicin-liposome-treated tumor was observed to be 0.33g, whereas the weight of the tumor treated with only PEG-liposome-doxorubicin and free doxorubicin was found to be 1.17 g and 1.38 g, respectively. **[106]**. Muthu *et al.* (2015) developed a theranostics micelle composed of D-alpha-tocopheryl polyethylene glycol succinate conjugated with Tf for the specific administration of docetaxel (therapeutic agent) and gold clusters (bioimaging module). A casting method was used to synthesize micelles, and then Tf was coupled to the surface of the micelles through carbodiimide chemistry. The formulated construct was tested in Tf overexpressed MDA-MB-231 cells and NIH-3T3 fibroblast cell line (no Tf overexpression). The flow cytometry study showed that the intensity of fluorescein (FITC) stained MDA-MB-231cells significantly shifted after being treated with formulated micelle. However, no significant shift was observed in the NIH-3T3 cells (FITC staining) treated with the same inducers. Further, Formulated micelle showed a 4-fold reduction in IC_{50}value in MDA-MB-231cells compared to NIH-3T3 cells. It is also observed that gold clusters showed a strong fluorescence signal with photostability property in the malignant cell imaging **[107]**. The Tf and NLBCs allow for improved therapeutic efficacy, prolonged circulation, and a better release profile, preventing non-specific binding with increased toxicity of the free drugs at the targeted site. However, certain drawbacks are observed while using a lipid system conjugated with Tf. Drug encapsulation and loading efficiency of Tf conjugated NLBCs were found to be decreased

as compared to non-conjugated nanocarrier. In addition to this, a long chain of PEG-polymer may not detach from the nanocarrier in the tumor microenvironment. Another drawback of employing Tf conjugated NLBCs is that the protein corona effect on Tf -NLCs may adversely affect Tf receptor targeting, thus slowing Tf -dependent absorption in the targeted region **[108]**. Also, high endogenous Tf levels in plasma, which saturate the Tf receptors in the blood-brain barrier, are another limiting factor in Tf-NLC accumulation in the brain. Because Tf-nanoparticle competes with endogenous Tf for receptor binding, the amount of Tf conjugated nanoparticle accumulation in the brain will be reduced **[109]**. Overall, the novel nanomedicines based on lipid and transferrin offer promising combinational cancer theranostics.

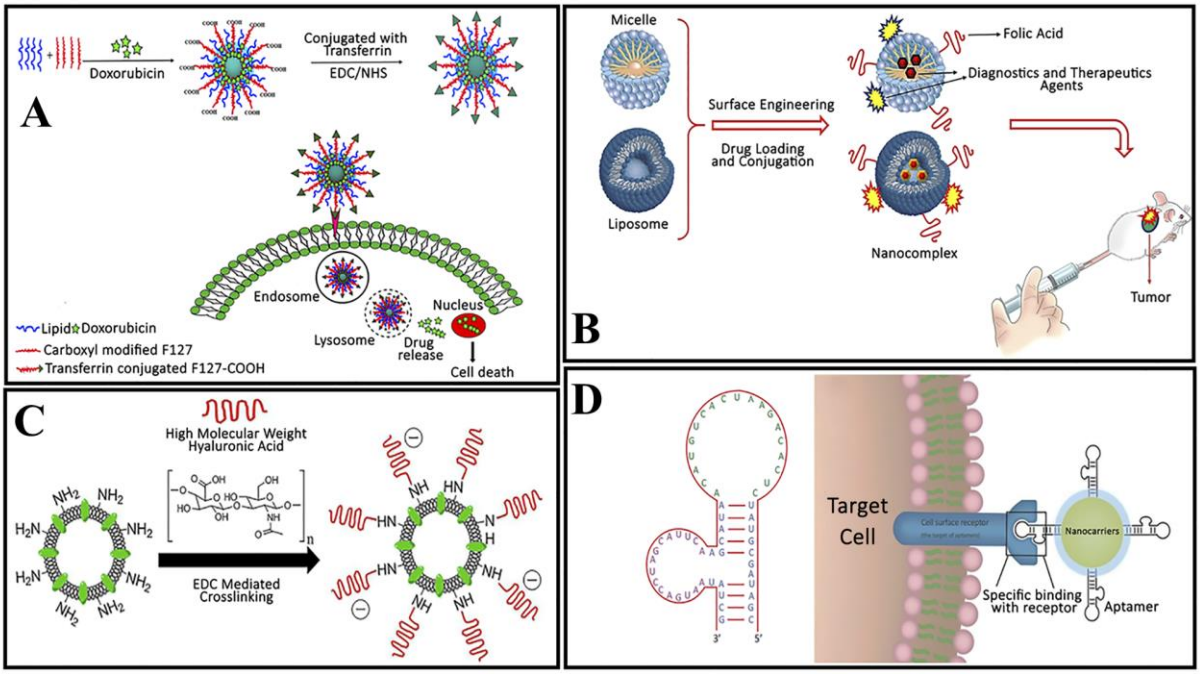

Figure 1.8. Schematic representation of ligand conjugated nanocarriers. (A)Synthesis of Tf conjugated nanocarriers and its interactions with cognate molecule (Reproduced with permission from **[110]**. (B) The FA conjugated drug-loaded micelle and liposome and their internalization in tumor-bearing mice. Reproduced with permission from **[111]**. (C)EDC-mediated crosslinking of HA on the surface of lipid nanocarriers. Reproduced with permission from**[112]**. (D) RNA-based aptamer and aptamer decorated nanocarrier and its interaction with the cognate receptor. Reproduced with permission from**[113]**.

6.2 Folate receptor

Folic acid is water-soluble, naturally found in many foods, and a key component in the production of nucleic acid. Folate has a high affinity to folate receptors(FR), expressed in 40% of human carcinoma, facilitating drug uptake by the receptor-mediated endocytosis **[114]**. Folate acid (FA) targeted drug delivery can be achieved by designing suitable linkers for specifically targeted cells to accomplish cytotoxic activities. Folic acid is a tiny substance with a 440 Da molecular weight stable over a diverse pH and temperature range. It is non-immunogenic and facilitates drug accumulation after conjugating with anti-tumor drugs or testing markers. The cells internalize the conjugated carrier through endocytosis, a non-invasive design for imaging cancer cells. The folic acid conjugated drug-loaded micelle and liposome with their internalization in tumor-bearing mice is well represented in **Figure 1.8B.** Egg phosphatidylcholine, cholesterol, cholesteryl hemi succinate, and DOPE are the main structural components of NLBCs (liposomes and micelles). The structural components of the liposome align in a spherical structure that has been formed either by a single lipid bilayer or multi-lipid bilayer when mixed with an aqueous solution. However, micelles are simple aggregates of lipids with a hydrophobic core. The outer surface of liposomes/micelles exposed with DOPE is exploited for conjugation with FA through covalent bonding. The FA-targeted liposomes/micelles demonstrated a 2.20-fold increase in cell toxicity compared to free drugs **[115]**. In various investigations, NLBCs were used as a linker for conjugating FA and anti-cancer drugs. Pawar *et al.* (2016) synthesized FA decorated SLNs (FA-SLNs) using an emulsification technique for tumor-specific delivery and targeting docetaxel and curcumin. Glyceryl monostearate, compritol 888 ATO, poloxamer 188, and PEG-stearic acid are the main structural components of the SLNs. In general, while preparation of the SLNs, oil is replaced by solid lipids (Phospholipid (90 NG)/Glycerol tristearate/Glycerol monostearate/Cetyl palmitate) in the emulsion, which improves the encapsulation efficiency stability of the system.

The EDC-NHS chemistry is used to conjugate FA to PEG-stearic acid. Formulation parameters such as stabilizer concentration, stirring speed, homogenization time, lipid concentration, stabilizer composition, and types of lipids used were optimized through a central composite design. The obtained nano construct was attributed to high drug encapsulation and desirable particle size with spherical morphology. Moreover, cellular uptake and tissue distribution studies were conducted on MCF-7 cells and Wistar rats. Fluorescence microscopy revealed that the FA-SLNs accumulated 36.84% more drugs compared to unconjugated SLNs inside the cells with minimum toxicity. Further, methotrexate at the dose of 15 µg/mL, when encapsulated in the FA-SLNs, induced toxicity to cancer cells and spared the normal cells. However, tissue distribution studies showed that docetaxel accumulation was maximum in the liver**[116]**. Yassemi *et al.* (2020) similarly utilized the FR for tissue-specific delivery of letrozole through FA-SLNs. First, SLNs were synthesized using structural components of tripalmitin glyceride, octadecyl amine, and the surfactant, Tween-80, by a solvent emulsification evaporation method. After that, the folate was conjugated with the amino group functionalized carrier through carbodiimide chemistry. The obtained nanocarriers were spherical with high encapsulation efficiency, and the drugs were dispersed into the amorphous structure. Moreover, the formulated nano construct was evaluated on MCF-7 to see the effect of cell toxicity. Results suggested that the efficacy in terms of IC50 of FA-SLNs was found to be 2.85 fold and 11 fold greater than unconjugated SLNs and free drugs, respectively **[117]**. The combination of FA and NLBCs, cytotoxicity, and selective permeability of NLBCs to pass through endosomal membrane indicates FA-NLBCs to be a powerful construct in the therapy and detection of various malignancies.

6.3 Hyaluronic acid

It is a polysaccharide consisting of repeating units of disaccharide N-acetyl-D-glucosamine and D-glucuronic acid through (β 1→3) bonds. Around 2000 repeating disaccharides are joined together by a (β1→ 4) glycosidic bond to form a massive polysaccharide. This molecule plays a huge role in migration, angiogenesis, proliferation, differentiation, and invasion by binding with specific receptors, e.g., CD44. Hyaluronic acid (HA) is a primary structural element of the cellular matrix and is considered an efficient drug delivery material **[118]**. Malignant cells such as pancreatic cancer, lung cancer, breast cancer, ovarian cancer, and prostate cancer overexpress the CD44 receptor on their cell surface **[119]**. It is a multifunctional transmembrane glycoprotein that plays an important role in tumor motility, self-renewal, and drug resistance and prevents apoptosis in cancer cells. More interestingly, CD44 is linked to self-renewal cancer stem cells; therefore, targeting it with a specific ligand-drug conjugate may destroy the cell population and eradicate cancer **[120]**. To achieve a dual role, such as tumor detection and removal of cancer stem cell-rich populations, targeting CD44 is a viable option for generating more successful cancer theranostics. The HA modulates the tumor microenvironment and subsequently promotes a malignant phenotype via interaction with the CD44 receptor, further inducing the intracellular signaling pathway **[121]**. The coupling of the HA to the surface of the NLBCs has the following advantages. Firstly, to begin with, HA on the outer shell of particles protects carriers while also regulating circulation time and bioavailability. Secondly, as the main structural element of the extracellular matrix, HA is biocompatible and prevents the nanocarriers from non-specific bindings with high efficiency. **Figure 1.8C** shows the amino expressed NLBCs(Liposomes) and their coupling with HA through EDC: NHS crosslinking. **[120]**. L-α phosphatidyl choline, DPPE, and cholesterol are the main structural components used to synthesize the liposome nanocarriers. The DPPE comprising the primary amine group has been utilized for preferential conjugation with the

carboxylic group of the HA through amide bond formation. The HA conjugated liposomes showed 4.48-fold more efficiency on CD44 expressed cancer cells compared to normal cells. Shen *et al.* (2015) have successfully utilized CD44 expressing cancer stem cells to design HA conjugated SLNs (HA-SLNs). Further, they tested the HA-SLNs for paclitaxel delivery to the CD44 expressing melanoma cells and mouse xenograft model. They found that the HA-SLNs induced apoptosis in the CD44 expressing cells and efficiently delivered the paclitaxel in to the melanoma lung tissue **[101]**. In an investigation, Glioblastoma multiforme (GBM), brain malignancy cell has been effectively targeted using HA decorated liposome. GBM cells that overexpressed CD44 have effectively accumulated doxorubicin, used as a potential anticancer agent compared to normal cells. The HA coupled liposome was tested against non-malignant cells (primary cortical astrocytes and primary microglia) and malignant cells (A-172). The comparative study found that HA conjugated liposome promoted preferential uptake of anticancer drug to A-172 cells with lethal concentration (LC50) of 0.114 ± 0.010 as compared to primary cortical astrocytes, LC_{50} of 0.511 ± 0.039 and primary microglia, LC50 of 0.317 ± 0.048. The aforementioned results suggested that HA conjugated liposomes selectively diagnosed and accumulated the drugs in the malignant cell **[112]**. In another fascinating study, stem cells with a high level of CD44 expression were successfully used to build an effective drug-nanocarrier construct. Initially, a delivery system based on stem cells that expressed strong phenotypes such as cancer stem cell-related marker, colony formation, and tumor outgrowth *in vivo* has been successfully developed. Further, the delivery system of hyaluronic acid conjugated paclitaxel-loaded solid lipid nanoparticles (HA-SLNs/PTX) has been synthesized and tested. Glyceryl monostearate, soy phosphatidyl choline, and cholesterol are main structural component of the SLNs. The electrostatic attraction approach was used to fabricate the HA-SLNs/PTX system. Dose dependent cytotoxicity of HA-SLNs/PTX and SLNs/PTX was assayed on B16F10-CD44+ and A549 cells. The IC50 value of HA-SLNs/PTX

(11.13 ± 1.62 µg/mL), followed by SLNs/PTX (18.11 ± 3.79 µg/mL) and then PTX (free) (31.39 ± 4.81 µg/mL) when tested these inducers on the B16F10-CD44+ cells. Similar outcomes were obtained in A549 cells, along with the IC50 value 23.99 µg/mL, 28.90 µg/mL, and 40.89 µg/mL, 2.72 µg/mL, induced with HA-SLNs/PTX, SLNs/PTX, and PTX (free), respectively. The obtained values suggest HA-SLNs/PTX detects carcinogenic cell line effectively and shows greater efficacy in terms of anti-cancer activity **[101]**. Overall, conjugating HA with NLBCs surface could be a practicable approach for detection and anti-cancer drug delivery. Moreover, circulation time, bioavailability, and enzymatic degradation of the anti-cancer drug can be enhanced significantly using HA mediated lipid formulations. It is also noticed that by selecting high molecular weight HA, the overall charge of the carrier remains unaffected and preferentially accumulated the drugs at the targeted site. Further the HA conjugated lipid nanocarrier when administered in blood circulation of the body, the conjugate swells up and creates cloud of long chain of HA which protects the carrier from opsonization from macrophage. The expression of genes involved in proliferation and inflammation is not induced by HA proving effective "bioinert" component of drug delivery systems. However, density of the HA on the surface of the lipid carrier is one of the important parameters which needs to be optimize since higher density increase the aggregation of NLBCs. However, long term stability and clinical translations are major drawbacks of the HA conjugated NLBCs.

6.4 Aptamer

Single-stranded deoxyribose or ribonucleic acid-based oligonucleotide is synthesized to bind with cognate molecules expressed in various types of melanoma cells. Different properties such as small size, tissue-specific penetration, high binding affinity, non-immunogenicity, and amenable modification make the aptamer a promising theranostics linker **[122]**. Once binding between the aptamer and cognate molecule is achieved, the nanocarriers enter the cells through

the plasma membrane. The aptamer may form various secondary structures because it makes self-complementary base pairs. The secondary structure of aptamer can arrange itself in a three-dimensional structure that further interacts with cell surface receptors through various weak bonds such as electrostatic attractions, van der Waals forces, π-π stacking, hydrophobic interactions, or even structure match **[123]**. Different types of chemical modifications such as the replacement of phosphorodithioate and 2^1 – o methyl in one nucleotide, replacement of phosphodiester backbone with boranophosphate of 2^1 – OH methoxy motif phosphothoate, reactive 2^1 – OH base of RNA functional group, incorporation of fluoro, 2^1 – OH amino protect the aptamer from cellular enzymatic degradation. Moreover, the non-bridging oxygen atom is substituted by one or more sulfur atoms which further enhances the stability of the aptamer **[124]**. On the other hand, DNA-based aptamer is highly recommended since it is resistant to 2^1 endonucleases. An S1411 is a 26-mer G-rich DNA oligonucleotide specific for a cognate molecule, nucleolin, overexpressed in various tumor cell types. The aptamer is useful in nanotechnology, neurosciences, medical imaging, and cancer targeted therapy because of its exceptional qualities **[125]**. NLBCs modified with aptamer play a huge role in cancer theranostics in this direction. During the preparation of various structural forms of NLBCs, an aptamer can be coupled with a lipid tail forming a three-dimensional structure. Multiple studies were reported using aptamer as a linker and NLBCs as a drug delivery carrier for successful theranostics of cancerous cells. The aptamer decorated liposomal nanocarrier and its interaction with the receptor is well illustrated and presented in **Figure 1.8D**. The EPOPC, cholesterol, rhodamine-DOPE, and DSPE-PEG2000-maleimide are the monomers used in the preparation of liposome nanocarrier using the lipid hydration method. Tris (2-carboxyethyl) phosphine hydrochloride is an efficient reducing agent used to reduce the AraHH001 aptamer and expose 5thiol. Through thiol reaction, the 5 thiols modified aptamer was further conjugated with C1 carbon of the maleimide functionalized on DSPE-PEG2000. The drug accumulation efficiency

using aptamer conjugated liposome was almost 3.8 fold higher than that of the PEG-liposome system, which suggested the potent detection of biomarkers **[126]**. Alshaer *et al.* (2015) developed the 2^1-F pyrimidine-containing RNA aptamer functionalized liposome for selective targeting of CD44 expressed tumor cells. The structural components of the liposome are DPPC, cholesterol, and DSPE-PEG-maleimide. Thiol-maleimide conjugation chemistry was used to conjugate 3'-thiol modified Apt 1 with maleimide functionalized to the surface of the liposome. Further, the intracellular distribution of the construct was evaluated using confocal microscopy for lung cancer cells and mouse embryonic fibroblast cells. Results suggested that the aptamer-liposome selectivity distributed the drugs compared to free liposome in tumor cells. Further, when A549 and MDA-MB-231 cells were treated with Apt1 conjugated liposome instead of un-conjugated liposome, the mean fluorescence intensity of both the cells increased significantly. There was no significant change in mean fluorescence intensity between Apt1 conjugated liposome and unconjugated liposome with NIH/3T3 negative cells **[127]**. In another interesting study, non-small cell lung cancer (NSCLC) was targeted using an aptamer conjugated lipid-polymer hybrid system (ALPHS). The hybrid system was synthesized using glyceryl monostearate, thiodiglycolic anhydride, lecithin, Poly(L-lactide) (5000)-poly(ethylene glycol) (2000)-maleimide (PLA-PEG-MAL) by a one-step precipitation method and loaded with docetaxel (DTX) and cisplatin (DDP), a potential anticancer drug. A549 cell-binding aptamer (S6, sequence: GTGGCCAGTCACTCAATTGGGTGTAGGGGTGGGGATTGTGGGTTG) with a sulfhydryl group was conjugated with PLA-PEG-MAL component through thiol reaction. The release kinetic pattern of constructed hybrid system is significantly improved which facilitated the sustained rate of drug release. DTX/DDP-ALPHNs had significantly improved cytotoxicity by 50% , and tumor inhibition ability by 81.4% as compared to non-ALPHNs and single drug loaded LPHNs. **[128]**. This selectivity of aptamer was found to be a 10-fold higher than that of the antibody and they first recognize the cell

surface then facilitates cellular internalization. However, Serum stability, renal filtration, and endocytic escape are all typical limitations of using aptamer as a ligand. Other disadvantages include a lack of diversity in the aptamer library, vulnerability to nucleases, and quick degradation in blood circulation. Further, the long chain of PEG-polymer causes the steric hindrance on the nanocarrier surface which reduces the cellular-based uptake of the drugs. Despite several efforts for producing efficient aptamer-based nanomedicine, only a small percentage of nanomedicines have been successfully utilized in clinical applications. Therefore, aptamer-drug-NLBCs techniques should be expanded, and in this direction, smart or environment-responsive linkers could help in detecting and accumulating the drugs in précised manner.

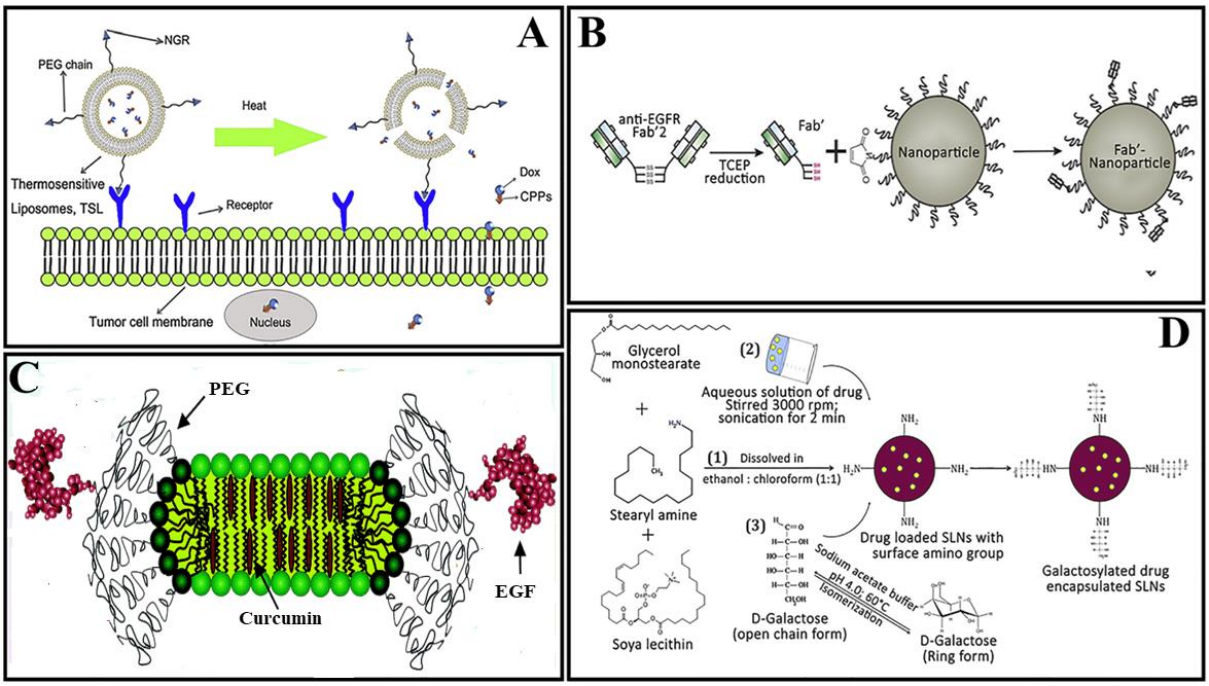

Figure 1.9. Schematic illustration of ligand conjugated nanocarriers. (A) NGR- conjugated thermosensitive liposome containing CPPs-DOX for detection of receptor and enhancing the DOX biodistribution. Reproduced with permission from **[129].** (B) Schematic representation of the synthesis of Fab' conjugated nanoparticle. Initially, reduction of anti-EGFR Fab'2 to Fab' fragment through Tris/2-carboxyethyl phosphine hydrochloride (TCEP) and generating three active thiol groups (-SH), which further react with maleimide group expressed on the nanoparticle surface. Reproduced with permission from **[130].** (C) EGF expressed nanocarrier for cancer theranostics of curcumin and doxorubicin in A-431 tumor cells. Reproduced with permission from **[131]**. (D) Step by step synthesis of galactosylated conjugated drug-loaded SLNs. Initially ring-opening of galactose molecule and further cross-linked with the amino group of stearyl amine exposed on the surface of SLNs through the EDC/NHS bioconjugate chemistry. Reproduced with permission from **[102]**.

6.5 Peptide

The peptide is a chain of oligo amino acids. The carboxylic group of one amino acid makes a covalent bond with an amino group of another and has great potential in the field of theranostics. Presently, the market value of peptide-based nanomedicines is presumed to be US$40 billion/year of the total pharmaceutical market **[132]**. The continuous emerging medicines market will contrive an even larger contributor shortly. Although small molecule-

based nanomedicine is presently the market's largest contributor, peptide-based nanomedicine has emerged with greater specificity because of multiple contact points with the cognate's receptors. Therefore, many researchers have engineered NLBCs using peptides as a linker for anticancer drug delivery and detection of cancer cells as well. Many peptides, such as LyP-1, K237, RGD (arginine–glycine–aspartic acid), and bombesin, are employed to adorn the NLBC surface to direct the overexpressed biomarkers on various tumor progressions such as melanoma, breast, and ovarian cancer, etc. **[133,134,135]**. The RGD peptides are well recognized for their ability to function as a strong recognition motif for a variety of integrins, such as αvβ3 and α5β1, found on cancer cell surfaces. These surface receptors are overexpressed in the tumor cells, and therefore, RGD peptide is used as a linker in numerous engineered NLBCs for cancerous theranostics. Xiong *et al.* developed a stabilized RGD-coupled liposomal vesicle (RGD-LV) for doxorubicin delivery and compared its performance with liposomal doxorubicin and free doxorubicin *in vitro* and *in vivo* conditions. Flow cytometry study demonstrated that RGD decorated liposome induced more drug accumulation with comparatively enhanced intracellular uptake in B16 and A375 cells. Yang *et al.* simultaneously examined the cumulative effect of two anti-cancer drugs, doxorubicin, and 5-fluorouracil in PC-3 human prostate carcinoma. They used a G3-C12 linker to modify the surface of an acrylamide polymer nanosystem to anchor doxorubicin and 5-fluorouracil. In this system, hydrazone, a pH-responsive connector, was used to bind doxorubicin to the covalent nanocarriers. However, 5-fluorouracils were conjugated to the NH_2-PEG_{2000}-DSPE using oligopeptide. The constructed nanocarriers showed high cytotoxicity with significant drug accumulation in prostate carcinoma overexpressed galectin. Additionally, the formulation increases cytotoxicity because drugs' synergistic genotoxicity, caspase-3 activation, cell cycle arrest, and DNA damage, resulting in tumor progression inhibition in the *in vivo* system as compared to nontargeted nanocarriers **[136]**. Cell-penetrating peptides (CPPs) are a type of

cationic amino acid-based oligopeptides that can cross cell membranes and deliver cargo in a precise manner. Although the CPPs are not specific to any receptor, they are widely utilized as a linker to engineer the NLBCs in numerous malignancies. Poly-arginine is a cell-diffusing oligo-peptide with an optimal chain length of 8 arginine units that has been successfully employed to deliver anti-cancer drugs intracellularly. **Figure 1.9A** shows the design of a thermosensitive liposome (TSL) containing CPP-doxorubicin (CPP-DOX) conjugate, and its surface is decorated with NGR (Asparagine-Glycine-Arginine) for detection and delivery of doxorubicin to aminopeptidase N(APN/CD13) over expressed cancer cells. The MSPC, DPPC, methoxy (PEG-2000)-DSPE, DSPE-PEG2000-Mal) are the main structural components of the TSL. As the primary targeting and detecting moiety for the specific tumor cells, NGR was conjugated to the exposed terminal of the PEG chains on the surface of the TSL(NGR-TSL). However, CPP was conjugated with doxorubicin using EDC-NHS and employed as secondary targeting moiety encapsulated in TSL to enhance the biodistribution of the drugs. The encapsulation of CPP inside the TSL protects the peptide from enzymatic degradation in blood circulation. The presence of the PEG chain on the liposomal surface facilitates the carrier to be transported in the tumor environment and subsequently enhanced the permeability retention (EPR) effect. The size and the zeta potential of the final nano bioconjugate were increased because of peptide addition which shielded the negative charge produced due to the phosphate group from the liposome. The efficacy in terms of cell toxicity of CPP-DOX/NGR-TSL was 1.5 fold higher than CPP-DOX/TSL however, no significant results were observed on normal cells **[137]**. In one of the interesting studies, a peptide HVGGSSV was isolated from an in vivo screening of a phage-displayed peptide library due to its selective binding within irradiated tumors and engineered on the surface of the doxorubicin-loaded liposome since it had a high affinity for lung carcinoma. Thin-film hydration technique was employed for the synthesis of the liposome

using structural components of cholesterol, DSPC, and maleimide-PEG2000-DSPE.The cysteine-containing peptide was conjugated with a liposomal surface modified with maleimide. Near-infrared imaging was used to examine the biodistribution of the peptide-modified liposome labeled with Alexa flour 750 within the irradiated lewis lung carcinoma. The doxorubicin-targeted liposome exhibited a long circulatory half-life and increased doxorubicin deposition within tumors to the point where it lasted more than 20 hours after intravenous delivery. Also, the formulated liposome accumulated the doxorubicin level 2-fold higher as compared to free doxorubicin. Moreover, modified liposomes reduced tumor growth, enhanced blood and lymphatic vessel destruction, and increased apoptosis in a mouse-bearing carcinoma **[138]**. Peptides as potential ligands are attracting a lot of attention among active ligands because of their unique advantages, such as ease of preparation, low cost, and high potency. However, For the time being, utilizing peptides as active targeting ligands has some drawbacks. Peptide ligands typically have a shorter half-life, which could result in premature degradation and dissociation of the payload before it reaches the target site. Meanwhile, peptide ligands have lower binding affinity and selectivity than antibodies or proteins, which enhances the likelihood of off-targeting. In this case, phage display can be extremely useful in identifying novel peptides with higher selectivity. The combination of several peptide ligands or peptides with other types of ligands should be expected to be created in the lab, with the huge potential to improve therapeutic efficacy in the clinic. Despite indisputable progress, a large number of variables such as lack of homogeneity and complexity of the biological environment, and our relatively limited understanding of their regulating elements, various process parameters such as energy supply for the production of micro-emulsion, vessels size, impeller speed, temperature, agitation time, sonication time affect the nanomedicine production at industrial level, have put off the clinical implementation of theranostics systems.

6.6 Growth factor as a target

The ability of growth factors to target the cancer cell-expressed receptor has been successfully utilized. Cancerous cells overexpress numerous growth factors such as vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), and basic fibroblast growth factor (FGF-2), which specifically interacts with antibody/ErbB-2/HER2. Among these growth factors, EGFR has a great affinity for anti-EGFR; researchers are more interested in selecting it as drug delivery and diagnosis marker. **Figure 1.9B** shows the schematic illustration of anti-EGFR Fab conjugated liposomal nanoparticles synthesis. One of the structural components of the liposome is DSPE-PEG-maleimide is involved in making bound with the thiol group of reduced Fab' fragment with high efficacy. The binding efficacy in terms of IC50 (M) of Fab' fragment and liposome-Fab' fragment with EGFR target (sEGFR501.Fc.) was found to be 5.6×10^{-9}, 23×10^{-9}, respectively **[130]**. Because of the covalent linkage of the thiol-maleimide process, the conjugation remained stable for at least two months at 4 °C. The EGFR is a high molecular weight compound with an extracellular N-terminal antibody-binding domain, a hydrophobic transmembrane region, and an intracellular C-terminal tyrosine kinase domain. The antibody-binding part effectively binds with EGFR, resulting in dimerization and cellular internalization of the receptor via the tyrosine kinase signaling pathway or clathrin-mediated pathway. Several growth factor-based nanomedicines have been approved for preclinical trials based on promising research results *in vitro* and *in vivo* systems **[139]**. Researchers are interested in developing a nanocarrier using an epidermal growth factor as a native ligand for targeting EGFR-expressing cancer cells. Anti-cancer drugs such as carmustine, gemcitabine, doxorubicin, and paclitaxel have been widely transported using anti-EGFR linkers and lipid as carriers in various model systems **[140]**. The EGF is a short peptide chain and is made up of 54 amino acid residues with a molecular weight of 6 kDa. It is a small molecule compared to an antibody which enhances the choice as an anchoring molecule for the development of the

nanosystem. Ahlgren *et al*. explored the potential of EGF coupled with PEG-stabilized lipid nanodisks for anticancer drug curcumin delivery. The lipodisks were prepared using a dissolution of DPPC, cholesterol, NHS-PEG_{3400} (2:2:1) in the organic phase, dried, and subsequently hydrated with an aqueous solution at 60°C for 1 h. After that, EGF was conjugated to the lipodisks surface by replacing NHS-PEG_{3400}-DSPE with EGF-NHS-EGF_{3400}-DSPE in an aqueous solution **(Figure 1.9C)**. In vitro study revealed that EGF coupled lipodisks effectively bound with EGFR expressing A-431 cells and facilitated the 1.5-fold accumulation of drug compared to uncoupled lipodisks **[131]**. Lung cancer cells overexpress the EGF tyrosine kinases receptor, facilitating the inactivation of the MAPK/PI3K/Akt/STAT pathway, resulting in angiogenesis, metastasis, and resistance against chemotherapy. Majumder and Minko synthesized NLCs comprising a multi-component system which includes EFG-TK inhibitor, gefitinib, luteinizing hormone release hormone (LHRH, cancer cell detection moiety), paclitaxel (anti-cancer drug), rhodamine (imaging agent), and siRNA (targeted to mRNA encoding EGF receptor). Trilaurin (solid lipid), α-tocopherol (liquid lipid), DSPC (Emulsifier), DSPE-PEG-2000 (surfactant), DOTAP (cationic lipid) are the structural components of the NLCs. The DSPE-PEG-LHRH peptide was synthesized using catalyst triethyl amine and was added to the lipid solution while synthesis of the carrier. However, anionic SiRNA was coupled with cationic lipid -DOTAP whose linker was exposed on the surface of the carrier. The results demonstrated that this multi-component delivery system has much higher anti-cancer efficacy (5-10 fold) than individual components applied separately, which depicts the strong detecting ability of the system **[141]**. Overall, anti-EGFR Fab' and EGF conjugated NLBCs can bind to human tumor cells receptor with high specificity and selectivity. Importantly, the binding causes the nanocarrier to be internalized through the receptor. Further, the binding of the anti-EGFR Fab' fragment on the surface of the lipid carrier did not significantly affect the size of the final formulations. It is also noticed that when

antibody has been conjugated directly to the surface of PEG-NLBCs, the binding affinity with the targeted molecule decreases significantly. The fact that explains the above phenomenon is a steric hindrance produced by PEGylation which reduces the binding affinity of the conjugated molecules. Further, certain disadvantages are associated with anti-EGFR, such as expensive, poor pharmacokinetics, and tissue permeability and needing considerable effort to produce them. Moreover, like peptides, and antibodies used as targeting agents, EGF is also sensitive to harsh conditions. As a result, organic solvent exposure and ultrasound sonication, freeze thawing must be avoided while preparing these conjugates.

6.7 Galactose

Malignant cells overexpressing numerous receptors on their cell surface have a high affinity for protein molecules, and these receptors are commonly known as lectin receptors **[142]**. Lectin-mediated drug delivery uses endogenous ligands such as lactose, fructose, fucose, mannose, and galactose **[143]**. Among them, galactose is one of the most utilized ligands targeting lectin molecules. Its free hydroxyl group at the C1 position interacts with lectin molecules with high affinity. Wang *et al*. successfully utilized the asialoglycoprotein receptor present on the hepatocytes that can especially recognize terminal β-D-galactose residue. They utilized liposomal based nanocarrier using modified lipid components to efficiently detection and deliver of doxorubicin to asialoglycoprotein overexpressed hepatocytes. The structural components include, doxorubicin, liposomes modified with a novel galactosylated lipid (CHS-ED-LA), cleavable PEG-lipid (PEG2000-CHEMS), HSPC, were used to synthesis of different liposome nanocarriers. Namely, conventional liposome (CL), galactosylated liposome (GaIL), pegylated liposome (PEG-CL), pegylated galactosylated liposome (PEG-GaIL) are exploited to encapsulate the doxorubicin. The tumor weight in gram (g) of H22 tumor treated cells with various formulations, doxorubicin solution, CL-DOX, GaIL DOX, PEG-CL DOX, PEG-GaIL DOX were compared and found to be 0.362 ± 0.127 g, 0.198 ± 0.112 g, 0.22 ± 0.129 g, 0.114

± 0.048 g, and 0.062 ± 0.033 g, respectively. PEG-GaIL DOX significantly reduced tumors weight suggesting the formulation effectively detect the malignant cells. Jain et al., constructed galactose decorated solid lipid nanoparticles to improve target competency associated with an anticancer drug with a similar approach. The presence of lectin molecules on the surface of adenocarcinoma human alveolar basal epithelial cells enabled the galactose conjugated solid lipid nanoparticle to interact precisely. It also enhanced the bioavailability of the drug. The chemistry between solid lipids (Soya lecithin) containing stearyl amine and galactose molecule involved galactose ring-opening in the presence of acetate buffer at pH 4.0, at 60°C . Subsequently, galactose aldehyde was conjugated with an amino group of the stearyl amine through EDC-NHS chemistry, resulting in an amide bond formation between them **(Figure 1.9D).** The results of the cell cytotoxicity assay were backed up by quantitative cell uptake studies, which demonstrated a 1.5-fold increase in drug uptake when galactose conjugated SLNs were incubated with A549 cells; it had a greater absorption of drugs than SLNs and free drugs. The enhanced bioavailability of the anticancer drugs revealed the biomarker-based detection with selectivity through galactose conjugated SLNs **[144]**. The above studies revealed that the drugs incorporated into galactosylated NLBCs, compared to NLBCs alone, possess increased bioavailability, stability, solubility, and encapsulation efficiency with no side effects of the anti-cancer drug in the biological system. However, the addition of galactose on the surface of NLBCs decreases the zeta potential and percent encapsulation efficiency. At the same time, the size of the final nano-bioconjugate increases, suggesting the system's stability could be affected. Percentage encapsulation efficiency reduction of final nano bioconjugate might be due to the loss of the surface adsorbed functionality in the acetate buffer.

Despite several efforts, only a small percentage of lipid-based nanomedicine is approved for commercialization. One of the major hurdles in achieving a higher success rate in developing nanomedicines is the translational gap between animal and human species. This

gap has emerged due to the physiological and pathological disparity between these species. Moreover, patients' heterogeneity can also hinder the efficacy of nanomedicines and is a subject of research on how they interact with the actual diseased population in real-time. This knowledge could help design and formulate nanocarriers and propel the field forward.

7. Challenges in the scale-up of lipid-nanomedicines manufacturing: from laboratories to industries

The manufacturing of lipid-based nanomedicines from lab scale to industrial vicinities requires the control of its structures, sizes, composition, shape, pharmaceutical, and physicochemical properties. The progression of lipid based-nanomedicines is dependent on advances in manufacturing technology that allows for scalable processes that adhere to GMP (Good manufacturing practices) quality guidelines. GMP protects the integrity and reliability of methods and products by mandating thoroughly documented protocols for each procedure that affects the quality of the completed product **[145]**. There are various facets involved in scaling up a lipid-based nanomedicine from the laboratory to the market. Some of these are the generally regarded as safe (GRAS) status of the substance and its nature, large-scale balancing of multicomponent systems, *in vivo* biodegradability, and toxicological aspects linked with the shape and size of the nanoparticle, and large-scale balancing of multicomponent systems **[146]**. Before selecting solvents, materials, the technique for developing nanoparticles, the expenses, and the acceptance of the end product by physicians and patients, attention must be taken. When a laboratory process is scaled up, the desirable properties of nanomedicines might be altered and lost. In one study, in research on the scale-up of a nanomedicine manufactured using the emulsion technique, it was revealed that with a rise in agitation time and speed of the impeller, the particles size was lowered yet the entrapment effectiveness was not affected **[147]**. Thomas *et al*. compared three formulation processes viz. nanoprecipitation method, emulsion diffusion method, and salting-out at both level lab-scale and pilot-scale since

manufacturing is affected by the production process. They found that the nanoprecipitation method significantly affects the particle size and entrapment efficiency of the formulations at the pilot scale. They suggested that various process parameters such as energy supply for the production of micro-emulsion, vessel size, impeller speed, temperature, agitation time, and sonication time should be the same for laboratory and batch levels for uniform production of lipid nanomedicine **[148]**. Based on the used nanomaterial, the scale-up manufacturing technique must be verified by the regulatory authorities and be cost-efficient. For an effective scale-up, various changes to the production procedure must be made to ensure that the end product's efficacy and stability are maintained and that the toxicological factors stay consistent, even if there is batch to batch minor deviations **[149]**. Because the batches are intended for use in clinical studies, they must be closely monitored, and also, GMP must be observed. Though lipid-based formulations, available commercially have been developed using traditional techniques, generating final products, their translation to clinics from the lab is frequently hampered by challenges such as contamination of the residual organic solvents, the inadequacy of homogeneity, the complexity of the scale-up method from laboratories to large-scale production, and variable high-quality reproducibility of products **[150].** To address such problems, novel technologies such as AKVANO® technology, DepoFoam® technology, lipid multiparticulate systems, Pastillation, Lipopearl™, Lipidots®, etc, and others are a few stepping stones toward the construction of potential alternative techniques to solve such difficulties **[150].** These provide a simple method, large throughputs of lipid-based nanomedicines with highly desired physicochemical qualities and very minor residual solvents, minimal cost, as well as baffling versatility and basic adaptability, making them ideal expertise for pharmaceutical companies. Demonstration of the ability to transfer the technology to a contract manufacturing company or development facility is critical, in which a well-controlled, cost-effective, and scalable methodology can be defined to produce a high quantity

of batch sizes under good laboratory discipline and, eventually, GMP requirements. A few of the difficulties that require attention, according to Liu et al., are as follows: (i) The need for compartmentalizing raw material, intermediate product, waste, as well as the constructed product in a production setting (ii) However, unlike simple and large-scale formulation protocol, long and tedious manufacturing steps and minimal yield reactions should be prevented (iii) Cost-effective raw materials with high-grade should be ascertained and utilized (iv) Nano-specific attention on reliability, shelf-life, degradation and stability (v) Composition-specific engineering challenges, including cost-efficiency, limited shelf-life and temperature-sensitive considerations **[151].** In this context, only a few nanomedicines, such as Abraxane (paclitaxel/albumin nanoparticles, Abraxis Bioscience) and Doxil (doxorubicin pegylated liposomes, Janssen), have been able to scale up from preclinical laboratory size to the number and quality required for clinical trials **[148]**. As a result, after achieving success on the laboratory scale, the researchers and their community should focus more on large-scale industrial production. Effective experimental methods might give appropriate answers to the issues faced by nanomedicine scale-up **[152]**. Therefore, nanomedicines will be more widely marketed once scale-up is reached for the treatment of a variety of ailments.

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Objective and goals of the Thesis

The goal of the whole work is to develop a ligand conjugated nano lipid-based carrier for cancer theranostics. We have utilized the bio-receptors overexpressed on the cancer cells successfully for designing and developing the nanocarrier system. Based on the high affinity of the N-acetyl-D-glucosamine (NADG), BSA, and lactoferrin towards their cognate receptors, these have been selected as diagnostic agents. Such a nanocarrier system is designed to enhance the bioavailability, accumulation, and efficacy in terms of IC50 value of the free drugs, and reduced their toxicity as well. Further, we tried to improve the release kinetic pattern by selecting the appropriate composition, and combination of the lipid monomers and synthesized the nanocarrier in the controlled conditions. We have also attempted to enhance the overall characteristics such as encapsulation efficiency, size, charge, and polydispersity index of the developed nanosystem. The objectives are as follows.

Objective 1.

N-acetyl-d-glucosamine (NADG) decorated nano-lipid-based carriers (NLBCs) as theranostics module for targeted anti-cancer drug delivery

Objective 2.

Development of BSA conjugated on modified surface of quercetin-loaded lipid nanocarriers for breast cancer treatment

Objective 3.

Design and Development of Lactoferrin conjugated Lipid-polymer Nano-biohybrid for Cancer Theranostics.

Thesis Overview

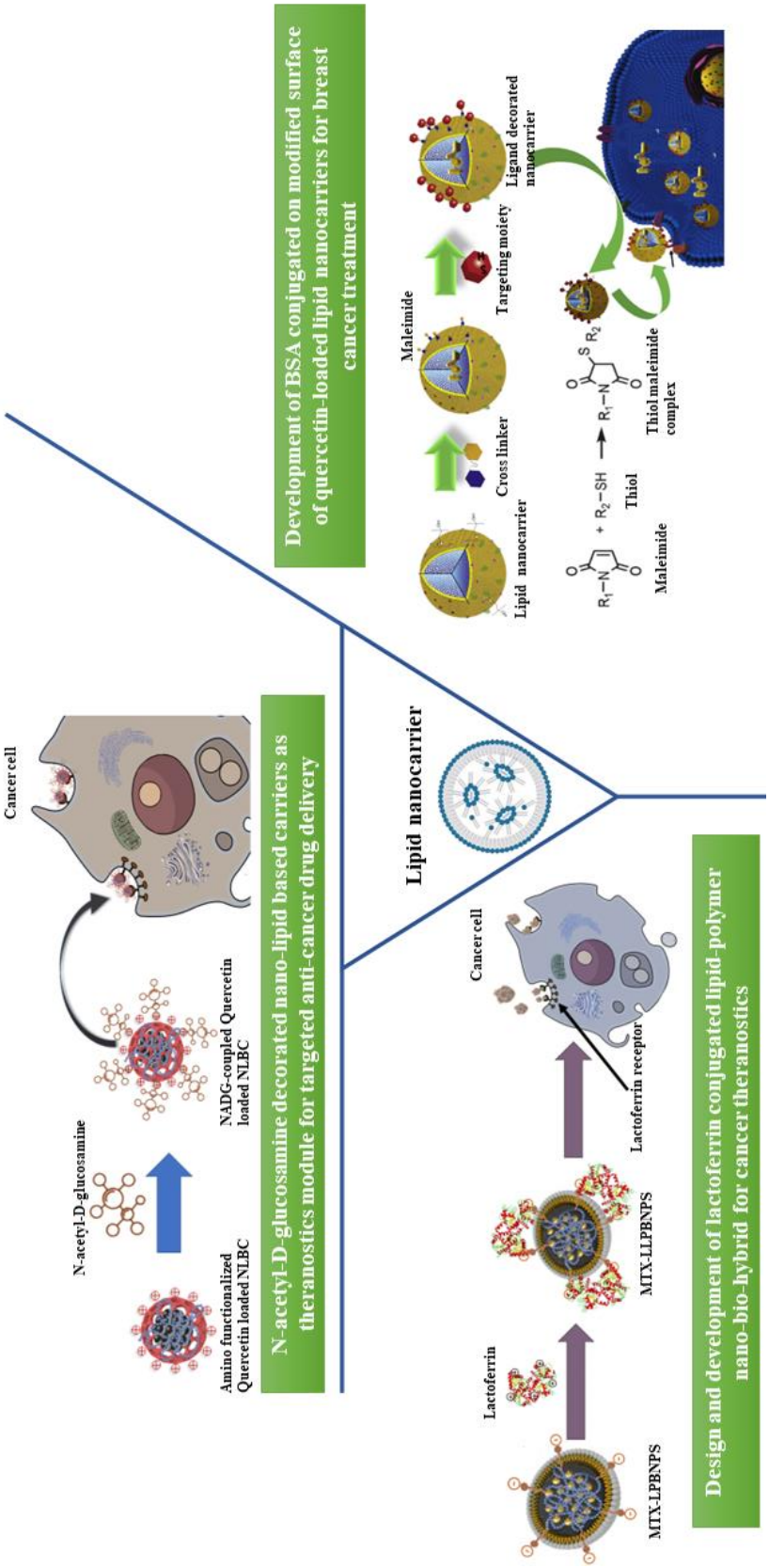

Figure: Pictorial representation of the thesis work