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Design, Development and Evaluation of Erlotinib-Loaded Hybrid Nanoparticles for Targeted Drug Delivery to NonSmall Cell Lung Cancer

Abstract

The objective of this work was to design, develop and evaluate erlotinib-loaded coreshell type lipid albumin hybrid nanoparticles (CSLAHNPs) for targeted drug delivery to nonsmall cell lung cancer (NSCLC). Erlotinib (ETB) is a highly selective, potent and reversible inhibitor of epidermal growth factor receptor tyrosine kinase (EGFR) which is overexpressed (50-90%) in NSCLC. ETB is marketed as film coated tablets for oral delivery. However, poor survival rate along with life-threatening adverse effects were reported from oral administration. Nanoparticulate delivery system of ETB might be advantageous to target the tumor cells, thereby increasing therapeutic efficacy and reducing off-targeting toxicities of ETB to healthy cells. In this work, a unique nanoparticulate carrier termed as CSLAHNPs was used for targeted delivery of ETB. The CSLAHNPs system was composed of albumin core and phospholipid bilayer shell. For active targeting to EGFR positive NSCLC, anti-EGFR half-antibodies (hAbs) were conjugate to EGFR expressing NSCLC. Overall hypothesis was to improve the efficacy of ETB in EGFR positive NSCLC using the targeted hAb-ETB- CSLAHNPs and untargeted ETB-CSLAHNPs. Blank CSLAHNPs were prepared by two-step method using bovine serum albumin and lipid mixture composed of 60:30:10 molar ratio of dipalmitoyl-phosphatidylcholine (DPPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) respectively. ETB was loaded into CSLAHNPs by incubation method. Murine anti-EGFR monoclonal antibody (mAb) was reduced to hAb using tris(2- carboxyethyl)phosphine and conjugated to maleimide terminated ETB-CSLAHNPs via maleimide-thiol conjugation reaction. CSLAHNPs were further characterized for physicochemical properties including mean size, polydispersity index, zeta potential, drug loading efficiency, in vitro drug release, and in vitro serum stability. The optimized ETBCSLAHNPs and hAb-ETB-CSLAHNPs were evaluated for their in vitro biological properties including cellular association cellular uptake, endolysosomal trafficking, cell viability, colony formation assay and western blots in two human lung adenocarcinoma cells; A549 (having wildtype EGFR) and HCC827 (having an acquired mutation in EGFR) cells. The mean size of hAbETB-CSLAHNPs (targeted) and ETB-CSLAHNPs (untargeted) was between 190-210 nm, suitable for intravenous delivery. The zeta potential, drug loading, and drug entrapment efficiency were about -13 mV, 2 % w/w, and 31% w/w respectively. CSLAHNPs exhibited sustained drug release profiles over 72-96 h in PBS pH 7.4. Fluorescent lipid tagged hAb-ETBCSLAHNPs showed enhanced uptake and accumulated in the cells. Significant reduction in % cell viability was observed for targeted hAb-ETB- CSLAHNPs compared to control groups in HCC827 cells after 72 h. The analysis of IC₅₀ demonstrated that both targeted hAb-ETBCSLAHNPs and untargeted ETB-CSLAHNPs could be more effective than ETB alone in both EGFR- positive NSCLC cells. Short-term stability data at refrigerator condition demonstrated that the lyophilized form of CSLAHNPs containing 16-fold sucrose (lyoprotectant) significantly improved the physical and chemical stability compared to liquid dispersion for 60 days of storage. Overall, the results indicated that hAb-ETB- CSLAHNPs and ETB-CSLAHNPs would be promising ETB delivery systems for EGFR-overexpressing NSCLC.

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Targeted Drug Delivery to Non-Small Cell Lung Cancer**

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Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Bivash Mandal
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DEDICATION

This dissertation is dedicated to my parents, Mr. Puspendu Mandal and Mrs. Rina Mandal and my beloved wife Chandrima Sinha for their unconditional love, care and constant encouragement to succeed.

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ABSTRACT

The objective of this work was to design, develop and evaluate erlotinib-loaded core-shell type lipid albumin hybrid nanoparticles (CSLAHNPs) for targeted drug delivery to non-small cell lung cancer (NSCLC). Erlotinib (ETB) is a highly selective, potent and reversible inhibitor of epidermal growth factor receptor tyrosine kinase (EGFR) which is overexpressed (50-90%) in NSCLC. ETB is marketed as film coated tablets for oral delivery. However, poor survival rate along with life-threatening adverse effects were reported from oral administration. Nanoparticulate delivery system of ETB might be advantageous to target the tumor cells, thereby increasing therapeutic efficacy and reducing off-targeting toxicities of ETB to healthy cells. In this work, a unique nanoparticulate carrier termed as CSLAHNPs was used for targeted delivery of ETB. The CSLAHNPs system was composed of albumin core and phospholipid bilayer shell. For active targeting to EGFR positive NSCLC, anti-EGFR half-antibodies (hAbs) were conjugate to EGFR expressing NSCLC. Overall hypothesis was to improve the efficacy of ETB in EGFR positive NSCLC using the targeted hAb-ETB- CSLAHNPs and untargeted ETB-CSLAHNPs. Blank CSLAHNPs were prepared by two-step method using bovine serum albumin and lipid mixture composed of 60:30:10 molar ratio of dipalmitoyl-phosphatidylcholine (DPPC), cholesterol, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) respectively. ETB was loaded into CSLAHNPs by incubation method. Murine anti-EGFR monoclonal antibody (mAb) was reduced to hAb using tris(2-carboxyethyl)phosphine and conjugated to maleimide terminated ETB- CSLAHNPs *via* maleimide-thiol conjugation reaction. CSLAHNPs were further characterized for physicochemical properties including mean size, polydispersity index, zeta potential, drug loading efficiency, *in vitro* drug release, and *in vitro* serum stability. The optimized ETB-CSLAHNPs and hAb-ETB-CSLAHNPs were evaluated for their *in vitro* biological properties including cellular association cellular uptake, endolysosomal trafficking, cell viability, colony formation assay and western blots in two human lung adenocarcinoma cells; A549 (having wild-type EGFR) and HCC827 (having an acquired mutation in EGFR) cells. The mean size of hAb-ETB-CSLAHNPs (targeted) and ETB-CSLAHNPs (untargeted) was between 190-210 nm, suitable for intravenous delivery. The zeta potential, drug loading, and drug entrapment efficiency were about -13 mV, 2 % w/w, and 31% w/w respectively. CSLAHNPs exhibited sustained drug release profiles over 72-96 h in PBS pH 7.4. Fluorescent lipid tagged hAb-ETB- CSLAHNPs showed enhanced uptake and accumulated in the cells. Significant reduction in % cell viability was observed for targeted hAb-ETB- CSLAHNPs compared to control groups in HCC827 cells after 72 h. The analysis of IC₅₀ demonstrated that both targeted hAb-ETB-CSLAHNPs and untargeted ETB-CSLAHNPs could be more effective than ETB alone in both EGFR- positive NSCLC cells. Short-term stability data at refrigerator condition demonstrated that the lyophilized form of CSLAHNPs containing 16-fold sucrose (lyoprotectant) significantly improved the physical and chemical stability compared to liquid dispersion for 60 days of storage. Overall, the results indicated that hAb-ETB-CSLAHNPs and ETB-CSLAHNPs would be promising ETB delivery systems for EGFR-overexpressing NSCLC.

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LIST OF ABBREVIATIONS

AchE	acetylcholinesterase
AFM	atomic force microscopy
ANPs	albumin nanoparticles
Apt	aptamer
ATP	adenosine triphosphate
BCA	bicinchoninic acid assay
BME	β -mercaptoethanol
BSA	bovine serum albumin
cc	cubic centimeters
CEA	carcinoembryonic antigen
CLSM	confocal laser scanning microscopy
CO ₂	carbon di-oxide
CSLAHNPs	core-shell type lipid albumin hybrid nanoparticles
CSLPHNPs	core-shell type lipid polymer hybrid nanoparticles
CYP3A4	cytochrome P450 3A4
DAPI	4',6-diamidino-2-phenylindole
DC-cholesterol	3 β -[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol
DDS	drug delivery system
DHA	docosahexaenoic acid
DL	drug loading
DLPC	1,2-dilauroyl- <i>sn</i> -glycero-3-phosphocholine
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
DOPE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DPPC	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine
DPTAP	1,2-dipalmitoyl 3-trimethyl- ammonium propane

DSC	differential scanning calorimetry
DSPC	1,2-distearoyl- <i>sn</i> -glycero-3-phosphocholine
DSPE-PEG2000	1,2-distearoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N (poly(ethylene glycol)2000)
DTT	dithiothreitol
Dtxl	docetaxel
ECM	extracellular matrix
EE	entrapment efficiency
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EPC	egg phosphatidylcholine
EPR	enhanced permeation and retention effect
ETB	erlotinib hydrochloride
ETB-ANPs	erlotinib hydrochloride loaded albumin nanoparticles
ETB-CSLAHNPs	erlotinib hydrochloride loaded core shell type lipid albumin hybrid nanoparticles
FBS	fetal bovine serum
FCM	flow cytometry
FITC	fluorescein isothiocyanate
FRAP	fluorescence recovery after photobleaching
FTIR	fourier transform infrared spectroscopy
gm	grams
h	hour(s)
hAb	half-antibody
hAb-ETB-CSLAHNPs	half-antibody conjugated erlotinib loaded core shell type lipid albumin hybrid nanoparticles
HCl	Hydrochloride
HER	human epidermal receptor
HPESO	hydrolyzed polymer of epoxidized soybean oil
HPLC	high performance liquid chromatography
HSA	human serum albumin
IgG	immunoglobulin G
kDa	kilodalton(s)

kV	kilovolt(s)
LPHNPs	lipid polymer hybrid nanoparticles
mA	milliampere
mAb	monoclonal antibody
Mal-ETB-CSLAHNPs	maleimide terminated erlotinib loaded core shell type lipid albumin hybrid nanoparticles
Mal-PEG-DSPE	1,2-distearoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (ammonium salt)
MAPK	mitogen-activated protein kinases
MDR	multi-drug resistant
MESNA	sodium 2-mercaptoethane sulfonate
MFI	mean fluorescence intensity
min	minute(s)
mL	milliliter(s)
mM	millimolar
MPS	mononuclear phagocytic system
MTOR	mammalian target of rapamycin
mTorr	millitorr
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
<i>n</i>	diffusion exponent
NaCl	sodium chloride
NBD-PC	1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}- <i>sn</i> -glycero-3-phosphocholine
nm	nanometers
NMR	nuclear magnetic resonance
NP	nanoparticle
NPs	nanoparticles
NR	not reported
NSCLC	non-small cell lung cancer
OQLCS	octadecyl-quaternized lysine modified chitosan
o/w	oil-in-water

PBS	phosphate buffered saline
PC	phosphatidylcholine
PCL	poly-epsilon-caprolactone
PCS	photon correlation spectroscopy
PDI	polydispersity index
PEG	polyethylene glycol
PEI	polyethyleneimine
PI3K	phosphoinositide 3-kinase
pKa	acid –ionization constant
PKB	protein kinase B
PKC	protein kinase C
PK/PD	pharmacokinetics/pharmacodynamics
PLA	poly-lactic acid
PLGA	poly(lactic-co-glycolic acid)
PMOXA-PDMS-PMOXA	poly(2-methyloxazoline)-block-poly(dimethylsiloxan)-block-poly(2-methyloxazoline)
PNPs	polymeric nanoparticles
PSMA	prostate specific membrane antigen
PXRD	powder X-ray diffraction
r^2	coefficient of determination
RBC	red blood cell
RES	reticulo-endothelial system
RGD	arginylglycylaspartic acid
rpm	revolutions per minute
RT	room temperature
SAXS	small-angle x-ray scattering
SCLC	small cell lung cancer
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
siRNA	small interfering ribonucleic acid
SOS	son of sevenless

T	temperature
TBST	tris buffered saline and tween 20
TCEP	tris(2-carboxyethyl)phosphine
T_e	eutectic temperature
TEM	transmission electron microscopy
T'_g	glass transition temperature
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
TM	trade mark
T_m	phase transition or melting temperature
TNF α	tumor necrosis factors
TPGS	D- α -tocopheryl polyethylene glycol succinate
Trp	Trypsin
UV	ultraviolet
VEGFR	vascular endothelial growth factor receptor
$W_{\text{Lipid}}/W_{\text{ANPs}}$	weight ratio of lipid to albumin nanoparticles
XPS	X-ray photoelectron spectroscopy

CHAPTER 1. INTRODUCTION*

Non-Small Cell Lung Cancer and Treatment Options

Lung cancer is a major health problem and the leading cause of cancer related mortality for both men and women worldwide (1). Every year about 1.59 million men and women die of this deadly disease around the world with around 169,000 deaths in the US alone (2, 3). Human lung cancer is divided into two major types based on the histopathological appearance: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC represents 85 to 90 % of all lung cancer cases, the rest being SCLC (4). The most common histological types of NSCLC include squamous cell, bronchoalveolar, and adenocarcinoma. Only 15 % of the cases are detected with cancer in the primary site while 22 % cases have lymph nodes metastasis and 55 % have distant metastasis at the time of diagnosis (5). There are various stages of the NSCLC; occult (hidden), stage 0 (carcinoma in situ), I (A and B), II (A and B), III (A and B), and IV.

There are different types of the treatments available for NSCLC; some treatments are standard or conventional, while some are investigational treatments being used in clinical trials. The treatment options include surgery, radiation therapy, chemotherapy, targeted therapy, laser therapy, photodynamic therapy, cryosurgery, electrocautery, chemoprevention, and new combinations therapy (6). NSCLC is treated with local resection and radiation therapy followed by chemotherapy. In approximately 85% cases, lung cancer patients are diagnosed with advanced inoperable stage of the disease (7), and the main treatment option is the chemotherapy.

Chemotherapy and Molecularly Targeted Therapy for NSCLC

In traditional or conventional chemotherapy, cytotoxic anticancer drugs are administered orally or intravenously. Depending on the stages of the disease, chemo drugs are used as neoadjuvant therapy (before surgery), adjuvant therapy (after surgery) or as main treatment (advanced stages). The traditional chemo drugs most often used in NSCLC include cisplatin, carboplatin, paclitaxel, docetaxel, gemcitabine, vinorelbin, irinotecan, etoposide, vinblastine, and premetrexed (8). In first line treatment of NSCLC at advanced stages, mono or combination chemotherapy is followed with a 4-6 dosing cycles of platinum based agent (cisplatin/carboplatin) alone or in combination with docetaxel, paclitaxel or gemcitabine. If platinum based therapy fails, second line therapy

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is used as the standard treatment option. Molecularly targeted therapy has become a routine option for the second line treatment for NSCLC. It includes 4-6 dosing cycles with one of the cytotoxic agents (vinorelbine, pemetrexed), or molecularly targeted agents (gefitinib, erlotinib, or bevacizumab).

Of all the second line treatments available, erlotinib (brand name Tarceva[®]; OSI Pharmaceuticals Inc., Melville, NY and Genentech, Inc. South San Francisco, CA) is the most widely prescribed drug. Erlotinib (ETB) belongs to epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) class of drugs (5). In 2004, US FDA approved ETB for the treatment of locally advanced or metastatic NSCLC after failure of at least one chemotherapy regimen. Chemically, ETB is N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine (**Figure 1-1**). The drug is available as ETB hydrochloride film coated tablets in 25 mg, 100 mg and 150 mg strengths (9). It has oral bioavailability of ~ 60%, median elimination half-life of ~ 36 h, apparent volume of distribution 232 L, and plasma protein binding of ~ 95%. It is metabolized primarily by CYP3A4 in liver. In a randomized, placebo-controlled, double-blind phase III trial (BR.21), ETB showed higher overall survival (6.7 months vs 4.7 months), higher response rate (8.9% vs <1%) (10), higher median duration response (7.9 months vs 3.7 months) and higher progression free survival (2.2 months vs 1.8 months) compared to placebo (10). Comparison between ISEL phase III trial for gefitinib (Iressa[®], AstraZeneca) and BR.21 trial demonstrated the survival benefit for all enrolled subjects with ETB compared to gefitinib which showed survival benefit for patients with adenocarcinoma or never smokers (11). In two phase III trials (OPTIMAL and EURTAC), significant progression free survival benefits were observed in patients with advanced EGFR-mutation positive NSCLC compared to standard chemotherapy (12, 13). With chemotherapy, 5-year survival at advanced stages (III and IV) of NSCLC remains only about 15 % and 1% respectively (14). One of the main reasons for the poor survival rates among NSCLC patients is the limited efficacy of traditional chemotherapy along with severe adverse side effects of the high doses of toxic anticancer drugs.

Erlotinib and Its Mechanism of Action

The schematic representation of the mechanism of action of ETB is shown in **Figure 1-2**. Epidermal growth factor receptor (EGFR) belongs to the bigger HER family of receptors. There are four subtypes of HER family receptors; HER1 (EGFR or erbB1), HER2 (EGFR2 or erbB2), HER3 (EGFR3 or erbB3), and HER4 (EGFR4 or erbB4). EGFR is a 170 kDa transmembrane glycoprotein receptor with an amino terminal extracellular domain (binds to ligands), a single hydrophobic transmembrane-anchoring region, a tyrosine kinase (TK) region and a carboxyl-terminal cytoplasmic region with tyrosine kinase activity (15). After binding with appropriate ligands (e.g. EGF), inactive monomers form active homo or heterodimers, results in the autophosphorylation of TK domain of EGFR. This process activates a complex network of downstream signaling pathways including MAPK, PI3k/PKB, PKC, RAS-RAF-MAPK p44/42. EGFR signaling involves cell maintenance, cell proliferation, survival, angiogenesis, and metastasis.

Figure 1-1. Chemical structure of erlotinib

Chemical name of erlotinib is N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy) quinazolin-4-amine.

Figure 1-2. Schematic diagram of the mechanism of action of erlotinib

EGFR (also known as HER1) have tyrosine kinase (TK) domains in the cytoplasmic portion of the receptor. Binding of ligands (EGF) to the extracellular domain of EGFR induces either homodimerization or heterodimerization of the receptors. Dimerization results in phosphorylation of TK domain leading to autophosphorylation, followed by intracellular signaling cascades, such as the RAS–RAF–MEK–ERK and PI3K–AKT pathways, that induce cellular proliferation, angiogenesis, and metastases. The binding of erlotinib to the ATP-binding site of TK domain results in blockage of the catalytic activity of the kinase, thereby inhibiting downstream signaling pathways responsible for cellular proliferation.

EGFR-TK has been identified as the effective molecular target and overexpressed (50-90%) in several cancer types including NSCLC (16). ETB is a highly selective inhibitor of HER1/EGFR-TK. The drug binds with the ATP-binding site of the tyrosine kinase domain of EGFR, which blocks the catalytic activity of the kinase, thereby inhibiting downstream signaling of the pathways responsible for cell proliferation, survival, metastasis, and angiogenesis (17).

Drawbacks of Erlotinib Therapy

ETB is administered as 25 mg to 150 mg oral tablet once daily. The oral route is most preferred and easy way of administering drugs for systemic therapy of NSCLC. However, a number of adverse effects or toxicities were reported. Severe skin rash and diarrhea were observed to be the major dose-limiting toxicities from traditional oral delivery of ETB (10, 18). Diarrhea occurred in 55% of the patients with severe diarrhea in 6% of the patients (10). Rash was reported in 75% of the patients (19). Other immune-mediated life-threatening toxicities include drug-induced hepatitis, Stevens-Johnson Syndrome(20), toxic epidermal necrolysis (21), gastrointestinal perforations (22), ocular lesions (23), interstitial lung disease (24), liver and kidney problems, bleeding and clotting problems.

Additionally, administration of oral ETB tablets is not convenient to cancer patients with gastrointestinal disorders or abnormalities. Approximately 40% of the cancer patients undergoing standard dose chemotherapy develop mucositis which causes structural and functional changes to the gastrointestinal tract (25). The pathological condition of gastrointestinal mucositis resulted in malabsorption of drugs and other nutrients (26). Evidently, intravenous administration of erlotinib was investigated to overcome the patient non-compliance of oral tablet. The phase I dose escalation study reported that a high dose of intravenous ETB formulation was well-tolerated with minor adverse events as compared to oral tablet (27).

Another major concern with ETB therapy is the development of drug resistance by the NSCLC tumors. ETB has been used as the first line therapy in patients with EGFR mutations consisting of either exon 19 deletions or L858R point mutation in exon 21(28, 29). However, most advanced NSCLC tumors develop resistance after 6 – 12 months of drug therapy (30). The main mechanisms of resistance identified include secondary resistance EGFR T90M mutations (31), oncogene kinase switch, and MET amplification (32).

Barriers to Drug Delivery in Solid Tumors

In order to reach the target tumor cells, intravenously administered therapeutic drug delivery systems must penetrate across blood vessel walls of the tumor and the tissue matrix or interstitium. The complexity of *in vivo* system was evidenced by the presence of multiple biological barriers that pose significant challenges in drug delivery

to solid tumors (33-39). The emergence of nanoparticles (NPs) based drug delivery systems showed potential advantages of efficient drug delivery to solid tumors in experimental therapeutics (40-42). However, their full clinical success still remains elusive with little to moderate improvement in clinical benefit (43). Only a small fraction of the drug reaches the tumor site, imposing significant risk for the development of drug resistance. After intravenous injection, NPs face the first barrier which is the mononuclear phagocytic system (MPS) or reticuloendothelial system (RES). MPS/RES is part of the immune system consisting of phagocytes cells such as monocytes and macrophages located in reticular connective tissues of liver, kidney, and spleen (44). The serum complement proteins such as C3, C4, and C5 form the corona around unprotected NPs, triggering the process of opsonization, followed by phagocytosis by circulating as well as residual macrophages (45). Opsonization process is influenced by physicochemical properties of the NPs such as size, shape, surface charge, and surface chemistry (46). One of the common ways to circumvent the MPS barrier is PEGylation that is coating of NPs by hydrophilic polyethylene glycol (PEG) or PEG-containing copolymers (47, 48). The bushy or mushroom structure of PEG chains repel the absorption of opsonin proteins on NPs *via* steric repulsion forces, thereby blocking the opsonization process (49). The second barrier is the vascular endothelial layer, a semi-permeable layer of cells which lines the inner walls of the blood vessels. The presence of proteoglycan layer (also known as glycocalix) which covers the endothelial layer, serves to control the permeability of solutes and macromolecules across blood vessels. The passage of molecules across healthy endothelial layer occurs by trans or paracellular pathways with size limit of 5-6 nm. However, the tumor endothelial layer is discontinuous with “gaps” in the lining which helps to extravasate NPs *via* the process known as enhanced permeability and retention (EPR) effect (50). However, significant differences were observed in tumor vascular morphology including pore cutoff size, organization, and blood flow rate not only among various tumor types but also within same tumor type. The heterogeneities of tumor vasculature have resulted in limited EPR effect of NPs (34, 51, 52). The third barrier is the tumor microenvironment. The solid tumor is characterized by an avascular necrotic core region, a hypoxic region, a vascularized circumference, and an acidic front (53). The extracellular matrix (ECM) or the tumor interstitium consists of collagen, elastin, proteoglycans, and hyaluronic acid (54). ECM provides resistance to the diffusion of NPs through the interstitium. Interstitial fluid pressure is higher in the center of the tumor and lower in periphery (52). The extravasation of NPs occurs mainly by diffusion and convection based on the concentration gradient or interstitial fluid pressure (54). Therefore, NPs has to overcome the outward fluid convection to diffuse into the tumor. Finally, NPs have to cross cellular barriers. The internalization of NPs into the tumor cells depends on interaction of NPs with cell membrane. The physicochemical properties of NPs including size, surface charge, hydrophobicity, presence of targeting ligands can influence their entry into the cells. Once internalized, NPs has to reach its intracellular target through endosomal vesicles and organellar barriers. Finally, overexpression of drug efflux transporter, P-glycoprotein (P-gp) may decrease the intracellular concentrations of drug inside the tumor. It has been demonstrated that NPs can overcome the P-gp-mediated efflux pump mechanism (55).

A lot of progress has also been made in the field of nanoparticulate drug delivery in order to overcome each of these barriers and a number of novel approaches have been brought to the forefront (34, 52, 56). Targeting tumor microenvironment was demonstrated to enhance drug distribution to tumors. Besides passive targeting by EPR effect, active targeting can improve chemotherapy retention in tumors. The potential of active targeting mediated drug therapy was demonstrated by FDA approval of trastuzumab emtansine (T-DM1, Genentech), an antibody-drug conjugate in the treatment of HER2 positive breast cancer (57). Normalization of the tumor microenvironment is another strategy to improve drug delivery in solid tumors. Vascular, stress and matrix normalization of the tumor microenvironment have demonstrated to enhance tumor localization and intratumoral distribution of chemotherapeutics. Vascular normalization using antiangiogenic therapy was shown to alleviate hypoxia and improve antitumor efficacy of NPs (58, 59). In stress normalization, targeting stromal cells and matrix molecules (collagen, hyaluronan) reduces stress by decompressing vessels and improves perfusion and drug delivery (60). Matrix normalization by degrading or modifying ECM can improve the delivery of nanomedicines to solid tumors (61).

Hybrid Nanoparticles for Erlotinib Delivery to Solid Tumors

Drug delivery system (DDS) plays an important role by influencing the pharmacokinetic profile of the drug, rate and duration of drug release, site of drug action, and finally the pharmacological effects and adverse effects (62). An effective DDS ensures that therapeutically active drug is available at the site of action at right concentration and time (63, 64). Therefore, an optimal DDS for ETB should improve the therapeutic efficacy of ETB by overcoming the barriers to drug delivery and also minimize toxicity, and lower the total dose required for the therapy. Nanoscaled targeted DDS meets most of the required attributes for the improved therapy of malignant diseases such as cancer. Rationally designed DDS with optimal physicochemical characteristics can reach to the tumor site passively through the leaky vasculature surrounding the tumor by enhanced permeation and retention (EPR) effect. Targeting ligand conjugated to the surface of nanoparticles (NPs) can achieve active targeting by binding to the specific receptors overexpressed by tumors. Surface modified NPs with flexible hydrophilic polymers have the ability to evade the RES system and prolong blood circulation time. NPs can carry high payload of anticancer drug (s), proteins, genes, and targeting ligands. NPs protect the entrapped drug from enzymatic inactivation. By reducing the free drug in circulation, the systemic toxicities could be minimized. There are various types of nanoscaled DDS which are approved or being investigated in clinical trials or in research and development phases. This includes liposomes, polymeric NPs, albumin NPs, nanocapsules, micelles, solid lipid NPs, dendrimers, PEGylated proteins, viral NPs, polymer-drug conjugates, antibody-drug conjugates, carbon nanotubes, and inorganic NPs (42).

The focus of nanoparticle design over the years has evolved toward more complex nanoscopic core-shell architecture using a single delivery system to combine multiple functionalities within nanoparticles. Core-shell-type lipid-polymer hybrid nanoparticles

(CSLPHNPs), which combine the mechanical advantages of biodegradable polymeric nanoparticles and biomimetic advantages of liposomes, have emerged as a robust and promising delivery platform (65-70). In CSLPHNPs, a biodegradable polymeric core is surrounded by a shell composed of layer(s) of phospholipids. The hybrid architecture can provide advantages such as controllable particle size, surface functionality, high drug loading, entrapment of multiple therapeutic agents, tunable drug release profile, and good serum stability (65). The multifunctional ETB-loaded CSLAHNPs conjugated with anti-EGFR half-antibody might be advantageous in selectively targeting EGFR overexpressed on NSCLC tumor and delivering ETB to increase therapeutic efficacy and reduce off-targeting toxicities of ETB after intravenous administration. The following section describes a comprehensive review on hybrid nanoparticles.

Review of Hybrid Nanoparticles Literature

Introduction

Nanoparticles (NPs) have attracted much attention because of their ability to deliver drugs to the therapeutic targets at relevant times and doses. Of all the common nanoparticulate systems, liposomes and biodegradable polymeric NPs (PNPs) have emerged as the two dominant classes of drug nanocarriers. This is evidenced by increasing numbers of clinical trials, research reports, and approved drug products (71-73). Both classes have advantages and limitations in terms of their physicochemical and biological properties. Historically, lipids have been used for several decades in various drug delivery systems including liposomes (71), solid lipid NPs (74), nanostructured lipid carriers (75), and lipid-drug conjugates (76). Most liposomes are biocompatible, biodegradable, non-toxic or mildly toxic, flexible, and non-immunogenic for systemic and non-systemic administration if their component lipids are from natural sources (77). However, there are several limitations of liposomal drug products from the viewpoint of physical and chemical stability, batch-to-batch reproducibility, sterilization, drug entrapment, and manufacturing scale-up (73, 77-79). Generally, PNPs are advantageous in terms of smaller particle size, tissue penetrating ability, a greater variety of preparation methods, availability of various polymers, improved stability in biological fluids, versatile drug loading and release profiles (72, 80). The limitations of PNPs include use of toxic organic solvents in the production process (81), poor drug encapsulation for hydrophilic drugs, drug leakage before reaching target tissues, polymer cytotoxicity, polymer degradation, and scale-up issues (80).

Novel, integrated systems known as “lipid-polymer hybrid nanoparticles” (LPHNPs) have been introduced in an effort to mitigate some limitations associated with liposomes and PNPs (69, 82). Briefly, the biomimetic characteristics of lipids and architectural advantage of polymer core are combined to yield a theoretically superior delivery system. LPHNs are solid, sub-micron particles composed of at least two components; one being the polymer and the other being the lipid. Various bioactive molecules such as drugs, genes, proteins, and targeting ligands can be entrapped,

adsorbed, or covalently attached in the hybrid system. The common choices of biodegradable polymers include polylactic-*co*-glycolic acid (PLGA), polycaprolactone (PCL), dextran, or albumin due to their biocompatibility, biodegradability, non-toxicity and previous use in approved products (83, 84). Lipids used are often zwitterionic, cationic, anionic and neutral phospholipids such as lecithin, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) or 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) (85-91). Various classes of LPHNPs are summarized in **Table 1-1** and are classified depending on the arrangement of lipid and polymer in the hybrid system. Due to their perceived advantages over other existing hybrid systems, significant effort has been directed towards the understanding of CSLPHNPs (92-101). The primary objective of this review article is to discuss CSLPHNPs which are composed of polymeric core and lipid shell. Discussions on other types of LPHNs will be limited as it is not within the scope of this communication.

Core-shell type lipid/polymer hybrid nanoparticles

CSLPHNPs continue to gain recognition in drug, gene, protein, and vaccine delivery (102-105). Based upon the CSLPHNPs concept, a new nanoparticulate drug delivery system, known as “Supra molecular bio-vectorTM” (SMBVTM), was introduced in the early 1990s by Biovector Therapeutics (106). SMBVTM is an artificial analog of virus composed of a modified polysaccharide hydrogel core covered with phospholipids acting as a shell. Because of its size (~ 60 nm) and architecture mimicking the structure of viruses (107), SMBVTM has been investigated for various purposes such as delivery of anticancer agents (108), nasal vaccines (107), and antisense oligonucleotides (109). Originally, core-shell type hybrid microparticles and NPs were synthesized with a lipid shell and a core that was made from inorganic materials such as silica (110), magnetic iron oxide (111), or organic materials such as polysaccharides (112), polystyrene (113), polyelectrolyte capsule (114), or polymer microgels (115). Comprehensive reviews by Troutier and Ladaviere (116) and Richter *et al* (117) are available on lipid membrane systems supported by various organic and inorganic colloidal solid cores and are not highlighted in this review. Instead, the main focus here is placed on polymeric cores (preferably biodegradable) which can be utilized in drug delivery systems.

CSLPHNPs systems can be described as the polymeric core coated with single or multiple layers of lipids that constitute the shell. Based on the concept of core-shell architecture, “lipoparticles” were first synthesized for various biotechnological and biomedical applications such as immunological kits and biosensors for amplifying biomolecular recognition (87, 89). The special features of lipoparticles are imparted by their method of preparation and use of the types of lipid materials. They are generally prepared by mixing liposomes and PNPs to form lipid-polymer complexes where a lipid bilayer or lipid multilayers cover the surface of the polymeric core. The space between polymeric core and lipid layer is occupied with water or aqueous buffer (**Figure 1-3**). Cationic or zwitterionic phospholipids have been used to construct the shell of the lipoparticles to promote electrostatic interactions with oppositely charged polymers.

Table 1-1. Various types of lipid polymer hybrid nanoparticles

Type	Description	Synonyms	Reference
Polymer core-lipid shell	Colloidal supramolecular assemblies consisting of polymer particles coated with lipid layers	Lipoparticles Lipid polymer particle assemblies Lipid-coated NPs Nanocell Polymer-supported lipid shells	(118), (90),(119), (89),(87, 88),(120), (121), (122), (123)
Polymer caged liposomes	These systems are composed of polymers, anchored or grafted at the surfaces of the liposomes to provide stability	Not applicable	(78, 79)
Monolithic HLPNPs	Lipid molecules are dispersed in a polymeric matrix	Mixed lipid polymer particles	(84)
Core-shell type hollow lipid-polymer-lipid NPs	Hollow inner core surrounded by concentric lipid layer, followed by polymeric layer, again followed by lipid layer along with lipid-PEG.	Not applicable	(124)
Erythrocyte membrane-camouflaged polymeric NPs	Sub 100 nm polymeric particles are coated with RBC membrane derived vesicles to mimic complex surface chemistry of erythrocyte membrane	Biomimetic NPs	(125)

Figure 1-3. Schematic diagrams of CSLPHNPs with its structural components
 CSLPHNPs with lipid bilayer (left) and monolayer (right).

In a recent report, Zhang *et al* (69) have designed a novel CSLPHNPs system that were composed of three functional building blocks each having distinct attributes that influences the whole hybrid delivery system. The first building block was polymeric core that was composed of a biodegradable hydrophobic polymer (e.g., PLGA) and acted as the carrier for poorly water soluble drugs. This core imparts controlled drug release from the system. The second component was the shell or the outer corona of the hybrid particles that was composed of hydrophilic substrates, most commonly lipid-PEG conjugates. This layer allows the particles to evade uptake by the immune system and imparts long-circulating characteristics. The shell can also be manipulated to facilitate the attachment of targeting ligands. Finally, the third component was composed of a lipid monolayer at the interface of core and shell. This layer helps to reduce drug diffusion from the core and water penetration into the core thereby increasing drug encapsulation and altering drug release rates.

Advantages of CSLPHNPs

The solid polymeric core of CSLPHNPs acts as a cytoskeleton that provides mechanical stability, controlled morphology, biodegradability, narrow size distribution, and high available specific surface area (126-128). The lipid shell enveloping the polymeric core is biodegradable, biocompatible and exhibits biomimetic behavior similar to cell membranes. The shell has the ability to interact with a wide variety of molecules, either within the membrane or on the surface of the membranes (129). Improved encapsulation of hydrophobic and hydrophilic drugs with therapeutically effective drug entrapment efficiency and drug loading have been reported in CSLPHNPs for a number of drugs compared to liposomes or PNPs (96, 99, 102). Amphiphilic character of lipids facilitates the adsorption of hydrophilic compounds on the hydrophilic bilayer surface and insertion of hydrophobic molecules into the hydrophobic lamellar region (129-132). This feature allows CSLPHNPs to entrap and deliver multiple hydrophilic and hydrophobic therapeutic agents simultaneously into the single delivery platform (99, 133). Optimization of the core and shell properties can result in tunable and sustained drug release profiles (134). CSLPHNPs exhibit storage and serum stability over prolonged periods (104, 134). Besides passive targeting of CSLPHNPs based on particle size, they can be conjugated with appropriate targeting ligands such as aptamers (134), folic acid (97, 135), transferrin (136), anticarcinoembryonic antigen half-antibody (94), single-chain tumor necrosis factor (121) to deliver NPs at the target tissues for treating cancers. Smaller than 100-nm particles (similar to virus-like architecture) are promising for intracellular drug targeting and vaccine adjuvants (137).

Methods of preparation of CSLPHNPs

Methods used to prepare CSLPHNPs broadly fall into two categories; the two-step method and the single-step method.

Two-step method. In this approach, the polymeric core and lipid shell are prepared separately using two independent processes; then the two components are combined by direct hydration, sonication or extrusion to obtain the desired lipid shell–polymer core structure (**Figure 1-4**). Several investigators have prepared lipid polymer particle assemblies or lipoparticles to obtain solid supported lipid bilayers which act as a model for artificial cell membrane and also for drug delivery applications (87, 89, 104, 119, 120, 133, 135, 138). In the two-step process, cationic lipid vesicles and anionic PNPs are drawn together by electrostatic interactions.(90).

The fusion of the PNPs and lipid vesicles can be accomplished by employing different mixing protocols (116). In such processes, the dry lipid film can be hydrated with the PNPs dispersion. Another alternative is to introduce the PNPs into pre-formed lipid vesicles. Such a process is usually followed by low energy mixing processes such as vortexing the resulting mixture. This mixture is heated at a temperature above phase transition temperature (T_m) of the lipid (89). This facilitates reorganization of the lipid onto the particle surface. The non-adsorbed lipids, micelles and free PNPs are separated by centrifugation to obtain a final CSLPHNPs dispersion.

There are several factors that affect final particle size of lipoparticles. Examples of relevant factors are methods applied to produce lipid vesicles (direct hydration, sonication or extrusion), mixing protocol of lipid vesicles/PNPs, type of polymers/lipids, pH and ionic strength of buffers used, surface charge of lipid vesicles, vesicle to particle ratio and temperature of incubation (89, 116). In general, addition of water or an aqueous buffered solution to dry lipid film forms large multilamellar vesicles. However use of additional steps such as sonication or extrusion leads to formation of small unilamellar vesicles with smaller particle sizes and a lower polydispersity index¹⁰. Troutier *et al* reported that particle of approximately 100 nm were obtained using membrane extrusion compared to 250 nm using hydration or 500 nm using sonication approach (89). Simultaneous loading of two drugs, doxorubicin and combretastatin, into “nanocells” using the two step approach has been reported (133). In this study, doxorubicin-PLGA conjugated PNPs were prepared using an emulsion-solvent evaporation technique. Then, combretastatin loaded lipid vesicles were prepared using phosphatidylcholine, cholesterol, and PEG-DSPE. Finally, hybrid dual drug loaded “nanocells” were obtained by extruding the mixture of PNPs and lipid vesicles. The size of the particles ranged from 180-200 nm. The authors reported that combretastatin released from the CSLPHNPs at a faster rate compared to doxorubicin. The differential drug release was attributed to localization differences inside “nanocells”. The authors postulated that combretastatin was entrapped at/in the shell, whereas doxorubicin was located in the core.

In the two-step process, the particle size and drug loading of the core can be precisely controlled to produce final lipid-polymer hybrid nanoparticles of appropriate size, drug loading and release characteristics (89, 133). In addition, the theoretical amount of the lipid required to uniformly coat the core with uniform bilayer of phospholipids can be calculated based on the properties of the core and phospholipids (139, 140). However, it should be cautioned that the two-step method may reduce drug encapsulation efficiency for water soluble drugs due to the incubation step since drug

Figure 1-4. Schematic representation of the steps involved in lipoparticle synthesis by two-step method

(a) Polymeric nanoparticle cores (PNPs) are prepared separately. (b) Lipid shells (liposomes) are prepared separately. (c) Both polymeric cores and lipid shells are mixed and incubated. (d) Finally, lipoparticles are obtained.

molecules may leak out from the core before a lipid coat is formed on the core surfaces (141). Some limitations of this method are the technical complexity and less efficient processes of preparing both polymeric core and liposome vesicles separately.

Single-step method. In an approach to circumvent the problems of time consuming preparation steps, a relatively simple approach which combines the dual steps of the two-step method into a single step has been evaluated. Here a nanoprecipitation process is synchronized with a simultaneous self-assembly process (**Figure 1-5**). One of the critical factors influencing successful preparation of CSLPHNPs using this method, is the amount of lipid for uniform lipid coating of polymeric core particles. Variations of the single-step method have been reported in the literature. These include modified solvent extraction/evaporation and nanoprecipitation methods.

Modified solvent extraction/evaporation method is a modification of the emulsion-solvent evaporation method first reported by Gurny *et al* (142). The method has been used to prepare CSLPHNPs loaded with docetaxel (86, 97) and paclitaxel (98). Briefly, the polymer and drug are dissolved in a water-immiscible organic solvent such as dichloromethane, chloroform, or ethyl acetate. Lipid is then dispersed in water by bath sonication, mechanical stirring, or sometimes with heat. The organic solution is mixed into aqueous phase and the resulting dispersion is sonicated using a probe sonicator in an ice bath. The dispersed phase is broken into tiny nanodroplets, which are solidified into nanospheres coated with lipid layer. The organic solvent is usually removed by evaporation in a rotary evaporator under reduced pressure or stirred overnight. The particle suspension is purified by centrifugation followed by washing. The washed particles are freeze-dried to obtain a dry powder. Liu *et al* (97) used this method to prepare folic acid-conjugated docetaxel-loaded CSLPHNPs having the particle size of approximately 200-300 nm and drug encapsulation efficiency of 60-66 %. They observed a decrease in mean particle size of CSLPHNPs with increasing concentrations of lipid. This phenomenon was attributed to the presence of DLPC lipid that acts as an emulsifier thereby lowering the surface tension of the lipid monolayer, resulting in lower surface free energy and smaller CSLPHNPs. A typical approach to entrap hydrophilic small and macromolecules in microparticles/NPs is to use a multiple emulsion/solvent evaporation method. A similar approach used to prepare hollow core-shell type lipid-polymer-lipid hybrid NPs. This method used a modified double emulsion/solvent evaporation for encapsulation and delivery of nucleic acids (124). The hollow aqueous core acted as the reservoir for hydrophilic small interfering RNA (siRNA). Briefly, in the first step, primary water-in-oil (w/o) type emulsion was formed by dispersing the aqueous siRNA into an organic solvent containing a polymer and a cationic phospholipid by sonication. Here the phospholipid layer helps to stabilize the aqueous droplets and increase the loading of siRNA by polycomplexation. A secondary oil-in-water (o/w) type emulsion was prepared by adding the primary emulsion into aqueous dispersion of another phospholipid (lecithin) and DSPE-PEG. Finally, the organic solvent was evaporated to prepare multilayered CSLPHNPs. Not only macromolecules but also water soluble hydrophilic small molecular weight drugs such as antibiotics have been encapsulated within CSLPHNPs *via* the double-emulsion solvent evaporation method (141).

Figure 1-5. Schematic representation of the single-step method involving nanoprecipitation and self-assembly processes

(a) Drug, polymer dissolved in organic solvent forming organic phase. (b) The organic phase is added dropwise into aqueous phase containing phospholipids. (c) The resulting dispersion is sonicated or homogenized to obtain CSLPHNPs.

In the modified nanoprecipitation method, polymer(s) and hydrophobic drug(s) are dissolved in a water-miscible organic solvent (e.g., acetonitrile or acetone). The organic solution is then added, drop by drop, to the aqueous dispersion containing lipid. The mixture is vortexed and subsequently homogenized or ultrasonicated to reduce the particle size to nanometer range. Valencia *et al* reported a similar method based on rapid mixing of lipid and polymer solutions using a continuous flow microfluidic device that used hydrodynamic flow focusing in combination with passive mixing structures to prepare CSLPHNPs in a single step (143). Their study indicates that in order to ensure proper dispersion of lipid and lipid-PEG conjugate, it is necessary to heat the aqueous dispersion (generally $\sim 65^{\circ}\text{C}$) before adding the organic solution. To uniformly coat the polymeric core with a lipid shell and to evaporate the organic solvent, the dispersion was stirred for several hours with a magnetic or mechanical stirrer. CSLPHNPs formed were purified by ultracentrifugation, centrifugal ultrafiltration or dialysis.

The critical factors to be optimized for particle size, polydispersity, and surface charge include the type of the lipid, ratio of lipid/polymer, phase-volume ratio of organic to aqueous phase and viscosity of the polymer (69, 93, 143). Docetaxel loaded CSLPHNPs were prepared by this method to produce particles of mean size of 66 nm and encapsulation efficiency of approximately 60 % (134).

Recently, a fast and simple method of producing CSLPHNPs using sonication was reported by Fang *et al* (93). They prepared CSLPHNPs of uniform and controllable size (~ 65 nm) and low polydispersity index (~ 0.08) by using bath sonication for 5 min compared to a few hours for other fabrication approaches. The size and polydispersity of the particles was effectively controlled by optimizing the ratios of lipid-PEG/polymer and lipid/lipid-PEG/polymer.

Drug loading and entrapment efficiency of CSLPHNPs

Many small molecular weight chemotherapeutic drugs, proteins and nucleotides have been encapsulated or entrapped in CSLPHNPs. One reason for poor drug loading (DL) and entrapment efficiency (EE) in CSLPHNPs is the presence of excess lipids could form vesicles by entrapment or adsorption of drug via hydrophobic interactions and/or hydrogen bonding (144). Additionally during purification, these vesicles are washed away, leading to drug loss. Therefore, the amount of the lipid required to uniformly coat the core nanoparticles has to be optimized using empirical and/or experimental techniques. There are various techniques for drug loading into CSLPHNPs. The drug can be loaded into both the polymeric core as well as in the lipid shell, thereby increasing the total drug payload. Moreover, two different drugs can also be loaded into the core and the shell (145). The most commonly used strategy is to incorporate the drug during core production or lipid film formation. Another option is to adsorb or absorb the drug with the cores and lipid vesicles separately before combining to form CSLPHNPs. However, the DL is generally expected to be better in the incorporation approach than the adsorption approach (146). The adsorption method has been used to load DNA into lipoparticles composed of PLA core/DPPC-DPTAP lipid shell (103). The

macromolecules or proteins shows greatest loading efficiency near its isoelectric point when it has minimum solubility and maximum adsorption (147). For small molecules, the use of ionic interactions between the drug and polymer can be an effective way to increase the drug loading (96). Examples of the factors which may influence the DL and EE are aqueous solubility of the drug, affinity and miscibility of the drug in both polymer and lipid phases (96), amount of lipid (144), drug-lipid charge interactions (141), aqueous phase pH (148), and methods of preparation. Often it is required to carry out in-depth physicochemical characterization during preformulation studies to optimize LC and EE (149). For instance, Li *et al* have analyzed the combined solubility parameters and partition coefficients for screening the best lipid and polymer for the highest LC and the maximum binding capacity to the cationic drug verapamil (149). Based on the approach, they have reported drug EE greater than 90% and DL between 5 to 36.1 % (96). The amount of the lipid is also a decisive factor for EE of lipophilic drugs in CSLPHNPs. Liu *et al* reported the decrease in EE from 42 % to 15 % when the lipid component was lowered from 0.1 % to 0.01 % for paclitaxel particles (144). Drug lipid charge interactions may be important for encapsulation of drugs. Cheow *et al* reported successful encapsulation of zwitterionic levofloxacin and ofloxacin when PLGA polymer and phosphatidyl choline (PC) lipid were used, whereas formation and loading of cationic ciprofloxacin into the CSLPHNPs was unsuccessful (141). When PC was replaced with non-ionic polyvinyl alcohol, ciprofloxacin loaded CSLPHNPs were successfully produced. The results suggested the possibility of unfavorable ionic interactions between the anionic PC and cationic ciprofloxacin for the failed formulation. The method of preparation also affects the DL and EE. The method used during core PNPs preparation, such as solvent displacement method leads to poor DL and EE for hydrophilic compounds (150). Another problem of the two-step method is that encapsulated drugs leak out before the lipid coat is formed (141).

Surface modifications of CSLPHNPs

In addition to the incorporation of drug, the outer surface of the CSLPHNPs can be functionalized to make long circulating particles along with the capability of active targeting. PEGs have become a standard for creating long-circulating NPs, thereby reducing plasma protein adsorption, macrophage uptake and particle aggregation, while increasing circulation time (151). For long circulating CSLPHNPs, outer surface is coated with hydrophilic polymeric chains of PEGs anchored in the bilayer with DSPE. The functional coating of PEG stabilizes the particles in storage due to steric hindrance by its long polymer chains (93). Another surface modification relates to the acidic environment of tumors. A pH-sensitive PEG coating shed its coating under the acidic condition, fused with cell membrane and entered into tumor cells (92). A red blood cell approach to particle surface functionalization was made by coating biodegradable PNPs with natural erythrocyte membranes. The membrane included both membrane lipids and associated proteins (125). The erythrocyte membrane which covered the polymeric core mimicked the natural endogenous erythrocyte, thus escaping from the recognition by the reticulo-endothelial system and producing a prolonged circulation time. Erythrocytes

have different surface antigens (blood groups) and patients should be cross-matched before injecting these “erythrocyte membrane camouflaged NPs”.

Surfaces of CSLPHNPs can also be modified with folic acid, monoclonal antibodies or therapeutic cytokines for targeting to tumors located in the various parts of the body. In general, antibodies or other targeting ligands are attached to the surface of liposomes and NPs using various covalent and non-covalent coupling techniques, as reviewed by Nobs *et al* (152). For example, anti-carcinoembryonic antigen (CEA) half-antibody was conjugated to the LPHNPs surface by a maleimide-thiol coupling reaction (94). CSLPHNPs containing attached folic acid on the surface can be prepared by using pre-synthesized DSPE-PEG_{5k}-Folic acid (97). Alternatively, CSLPHNPs can display a cell death ligand such as tumor necrosis factor α (TNF α) on the outer surface which mimics the bioactivity of membrane bound TNF α . In one study, the dual attachment of TNF α in both the core and the shell showed strong and specific binding to TNF receptor-expressing cells (121).

Physicochemical characteristics of CSLPHNPs

Several physicochemical and biological techniques for the characterizing CSLPHNPs are summarized in **Table 1-2**. Also included are discussions on mechanism of hybrid particle formation, structure and stability of CSLPHNPs.

Interaction and mechanism of hybrid particle formation. The interactions between lipids and polymer particles to form hybrid particles have not been well defined. Generally, different mechanisms of lipid-polymer hybrid particle formation can be distinguished based on the method of preparation. In the single-step method, polymer particle formation involves the precipitation of polymer from an organic solution and the diffusion of the organic solvent in an aqueous medium (93). Then, the lipid molecules self-assemble spontaneously by hydrophobic interactions on the polymeric particle surface to form a monolayer. In cases when the lipid-PEG component is incorporated, the lipid moiety of the lipid-PEG conjugate is inserted into the lipid monolayer, and the polar PEG moiety faces outward into the external media to form the stabilizing shell for the hybrid particles.

The possible mechanism of hybrid particle formation in the two-step method can be understood from a study by Carmona-Riberio and de Moraes Lessa (113). Their study involved phospholipid adsorption by polystyrene particles. According to the authors, the process occurs in two steps. First, the phospholipid forms a bilayer in aqueous solution and attaches to the polystyrene particle surface by adsorption to form homodispersed and stable phospholipid vesicle-covered particles. Second, after bilayer attachment, hydrophobic attractions between the polystyrene surface and hydrocarbon chain of the phospholipid bilayer collapses the bilayer structure and leaves a monolayer covering the polymer particle. In the process, the lipid and polymer contact is favored by electrostatic interactions, hydrophobic attractions, or van der Waals forces. In addition, the input of

Table 1-2. Summary of the techniques used for physicochemical and biological characterization of lipid-polymer hybrid nanoparticles

Parameter/property	Method of characterization
Particle size distribution	Photon correlation spectroscopy (94, 102, 134, 141, 153)
Surface charge	Zeta potential by PCS (102, 134, 153)
Morphology	TEM (86, 89, 102, 134), SEM (94, 141, 154), AFM (155), CLSM (89, 123), fluorescence microscopy (89, 134)
Lipid shell thickness	SAXS (87), TEM (87)
Interface chemical composition	XPS (89, 98)
Lipid shell fluidity	FRAP (123), fluorescent probes (106)
Lipid shell transition	NMR (87, 88, 96), FTIR (96), DSC (96), PXRD (96)
Drug loading and entrapment	HPLC (98), dialysis (86, 134), centrifugation (141), membrane filtration (102)
Drug release	Dialysis followed by HPLC(86, 134)/UV-visible spectrophotometry(96, 141), sample and separate method (98, 102)
<i>In vitro</i> cellular uptake	Fluorescence microscopy (94, 97, 134)
Cell viability and cytotoxicity	MTT cell viability assay (86, 98), MTS cell proliferation assay (134), trypan blue staining (102), clonogenic assay (102), ATPLite 1-step luminescence ATP detection assay(94)

external energy such as heating, sonication or agitation helps to rearrange lipids onto the polymer particles. Surface charges also play a major role in forming the lipid layer onto polymer particles. Stable particles are formed by electrostatic interactions between a negatively charged polymer and a cationic lipid. Moreover, affinity of the phospholipid for the polymer particle depends on the hydrophilicity of the polystyrene surface. Surface hydration of the polystyrene particles can shield the attractive forces and decreases affinity for the lipid monolayer coverage.

Structure of CSLPHNPs. The morphology, two dimensional fluidity, lipid shell permeability, and distribution of lipids in polymeric particles have been assessed using confocal laser scanning microscopy and cryo-transmission electron microscopy (Cryo-TEM) (123). Often, samples in TEM are stained with uranyl acetate, osmium tetroxide, or phosphotungstic acid for better imaging contrast to differentiate the core-shell structure. Zhang and co-workers deciphered the structure of the PLGA-lecithin-DSPE-PEG LPHNs by TEM using negative staining of the low electron dense lipid layer (69). Information about the structure of the hybrid particles are obtained by using conventional fluorescence microscopy and confocal laser scanning microscopy (CLSM). For example, the co-existence of a polymer core and lipid layer has been confirmed after overlay of the fluorescent images of nitro-2-1,1-benzoxadiazol-4-yl phosphatidyl choline (NBD-PC) at 365 nm for the polymeric core and at 534 nm for the lipid layer (89). As indicated previously, lipid composition and its concentration play a significant role in the formation of various nanostructures of hybrid particles. Thus, the presence of excess lipid during preparation leads to the formation of multilamellar lipid coatings on the particle or may form free liposomal vesicles. Bershteyn *et al* (123) reported two distinct structures when an excess concentration of lipid (DOPC) and lipid-PEG conjugate (DOPC-PEG) were used to prepare lipid/PLGA hybrid NPs. In the first case when excess DOPC was used, it formed an onion-like structure with multilamellar stacks of lipid packed together around the polymer core. When 10% mole of DOPC was replaced by DOPC-PEG, lipid “flowers” were formed with “petals” extruding from the polymer core.

Stability. Evaluation and optimization of physical or colloidal as well as chemical stability are required for any nanoparticle based drug delivery system to prepare a stable product with an acceptable shelf-life. The phospholipids that constitute the shell of the CSLPHNPs may act as surfactants to stabilize the hybrid NPs (156-158). Often times, the phospholipids alone are not enough to stabilize the system. For instance, the electrostatic repulsion between colloidal particles failed to stabilize a hybrid nanoparticle based system prepared from poly(lactic acid) core and lipid mixtures composed of DPPC/DPTAP when incubated in high ionic salt concentration such as 10 mM aqueous salt solution (159).

Four major factors that affect the colloidal stability of lipoparticles have been identified. These include pH and ionic strength of the aqueous medium, temperature, curvature of radius of lipoparticles, and vesicle-to-particle ratio (87). These factors are discussed below.

Lipoparticles usually become unstable with an increase in ionic strength of the continuous phase. For lipoparticles composed of a poly-lactic acid (PLA) core and DPPC/DPTAP lipid shell, a significant increase in particle aggregation was seen when the ionic strength of the aqueous phase increased from 1 mM to 150 mM of NaCl solution. This phenomenon can be explained as ion screening of electrostatic charges on the particle surface (160). The adsorption of lipid onto polymer particle is affected by incubation temperature. When incubation occurs at temperature (T) below glass transition temperature (T_g), the entire vesicle adheres onto particles without rupturing. However, when T is greater than T_g , lipid reorganization onto the polymer particle is accelerated. Sicchierolli and Carmona-Riberio (161) studied the adsorption of DPPC lipid on the surface of polystyrene microspheres at two different temperatures (25°C and 65°C) for 1 h. High adsorption of DPPC at room temperature suggested entire vesicle adhesion on latex particles. But at a temperature above T_g for the lipid, monolayer coverage on polystyrene particles was observed because of a change in the physical state of the lipid into a liquid-crystalline state. Spontaneous or intrinsic curvature of the lipid monolayer assemblies arises from the geometric packing of the lipid molecules by intermolecular interactions (162). Generally, small vesicles having a higher curvature radius tend to coat the smaller polymer particles (163). For spherically shaped monolayers, intrinsic curvature (R) of the lipid monolayer membrane can be derived from the **Equation 1-1**:

$$1/R = [2(V/Al - 1)/l]/[1 + \sqrt{(4V/Al - 1)/3}] \quad (\text{Eq. 1-1})$$

Where V is the volume of the entire lipid molecule, l is the length, and A is the area of the lipid head group at the lipid-water interface.

The proportion of lipid vesicles with regard to polymeric particles is an important parameter affecting overall size and stability of lipoparticles. Vesicle to particle ratio (A_v/A_p) can be expressed by the **Equation 1-2**:

$$A_v/A_p = \frac{\text{Total surface area of the lipid vesicles}}{\text{Total surface area of polymer particles}} \quad (\text{Eq. 1-2})$$

Where A_p can be determined from the particle number and mean diameter. Based on the study by Troutier *et al* (90), it can be hypothesized that the stability of the lipoparticles depends on the value of A_v/A_p . For instance, a high A_v/A_p value suggests electrostatic stabilization of the lipoparticles; while a low A_v/A_p value suggests aggregation will occur. The aggregation behavior at low A_v/A_p values can be attributed to the formation of bridges between lipid and polymer and incomplete coating that exposes the anionic zone of the polymer.

One approach to improve the colloidal stability of CSLPHNPs is by steric repulsions between particles after incorporating a lipid-PEG conjugate into the formulation (164, 165). It has been reported by Thevenot *et al* that lipoparticle stabilization was improved drastically from 1 mM to at least up to 150 mM sodium chloride solution for a period of 1 year at 4°C when 10 mol % lipid-PEG conjugate was added into the formulation (159). In the process of stabilization by lipid-PEG conjugate,

two important aspects were identified; PEG degree of polymerization (n) and molar percentage of lipid-PEG conjugate, which affected the final stability of lipoparticles. The stability of lipoparticles towards ionic strength revealed that longer the PEG degree of polymerization (i.e. chain length), the greater the stability in polar salt solution. The decreasing order of lipoparticle colloidal stability was reported as a function of PEG degree of polymerization: PEG₁₁₃ > PEG₄₅ > PEG₁₆. The molar percentage of lipid-PEG conjugate also affected the amount of lipid adsorbed onto particles, thereby affecting the surface coverage by PEG. The amount of lipid-PEG adsorbed decreased when n increased. Due to the steric hindrance by long PEG chains, lipid-PEG₄₅ conjugate adsorption was 3 mol % compared to initial amount of 10 mol %.

Another approach to improve the colloidal stability of CSLPHNPs is to incorporate suitable amounts of additional surfactants along with the phospholipids (141). For example, addition of 10 % of D- α -tocopherol polyethylene glycol 1000 succinate, TPGS (an amphiphilic biocompatible, biodegradable surfactant) along with PC confers stability of CSLPHNPs in phosphate buffered saline (141). Reasonably, the projection of the long and bulky PEG chain of the TPGS enhances stability as compared to small choline head group of PC (166). Finally, lyophilization may be used to further enhance the colloidal stability of CSLPHNPs in storage (167).

Unlike the physical stability issue that is a common concern for CSLPHNPs dispersions, the chemical stability is drug specific depending on the presence of susceptible functional groups and also the aqueous solubility of the compound. For example, drug molecules containing esters and amides are susceptible to hydrolytic degradation, while oxidative degradation is common for amine compounds (168). For poorly water soluble drug molecules, the possibility of chemical reactions in CSLPHNPs is not as substantial as that in solution-based formulations. Considering the inactive ingredients of CSLPHNPs, the phospholipids may degrade by hydrolysis and oxidation reactions during their storage in aqueous dispersions (169). The common strategy to enhance the chemical stability of CSLPHNPs is to transform the nanoparticles dispersion into dry solid dosage form using lyophilization technique with suitable cryoprotectants (167, 170).

Immunocompatibility of CSLPHNPs

Drug delivery systems including CSLPHNPs should be biocompatible, hemocompatible and immunocompatible thus avoiding undesirable interactions with the immune system (171). The recognition of therapeutic nanoparticles as foreign entities may result in multilevel immunological responses (e.g. cytokine release, interferon response and lymphocyte activation) leading to severe toxicity and/or lack of therapeutic benefit (172).

Since a CSLPHNPs system is composed of polymeric core nanoparticles and lipid shell, the immunocompatibility properties of the individual components should be considered. There is ample evidence showing the immunogenic properties of polymeric

nanoparticles composed of synthetic polyesters and polyanhydrides (173, 174). Although, the phospholipid bilayers are made up of natural phospholipids found in body, it has been reported that therapeutic liposomes containing paclitaxel, or docetaxel activate the complement system resulted in adverse immune phenomenon C activation-related pseudoallergy (175, 176). Complement activation can be enhanced by the physicochemical properties of liposomes including size (177, 178), charge (179), aggregation (180), polyamino coating (181), presence of endotoxin contaminants (182), drugs like doxorubicin (180), and PEGylation (183). Liposomes are vulnerable to immune recognition since the vesicles mimic the size and shape of some pathogenic microbes, ectosomes, nanobacteria, and viruses. Additionally, lack of self-discriminating molecules (e.g. C control proteins) on the phospholipid bilayers makes them susceptible to immune attack (171, 184-186). Currently there is scarcity of immunocompatibility studies on the CSLPHNPs and detailed investigations are warranted. The pioneering work on the immunological characteristics of CSLPHNPs including complement system activation, plasma/serum protein binding and coagulation cascade activation was reported by Salvadore-Morales *et al* (153). Among the three surface functional groups of CSLPHNs tested, the methoxy group induced lowest level of complement activation compared to the amine and carboxyl groups. They showed that the surface chemistry of the CSLPHNPs also changed human plasma and serum protein adsorption profiles. The findings of the complement activation and coagulation assay of their study provided evidence for good biocompatibility of CSLPHNPs.

Based on the immunocompatibility issues of polymeric core NPs and liposomes, it is necessary to evaluate the immunocompatibility properties of the CSLPHNPs. Several *in vitro* and *in vivo* techniques such as complement activation assay, platelet count and function test, plasma coagulation, and protein binding studies are available (171, 187). Assessment of the complement activation proteins (e.g. SC5b-9, Bb, C4d) using enzyme-linked immunosorbent assay is one of the most useful *in vitro* predictors of the immunological reactions (188).

The immunocompatibility of a complex drug delivery system such as CSLPHNPs is often challenging to predict based on their physicochemical properties due to the composition of formulations that differs both in nature and percentage of lipids and polymer. Moreover, the immunological response not only depends on the biomaterials but also on the host innate immune reactivity. Therefore, detailed investigations accounting for both delivery system and patient related factors should be performed to assess the immunocompatibility.

Applications of CSLPHNPs

Various applications of the CSLPHNPs are summarized in **Table 1-3**. Among their versatile applications, some major areas with significant clinical implications were discussed. These major application areas include vaccine adjuvants, cancer targeting and gene delivery. In cancer targeting, the most widely investigated disease areas include breast cancer, prostate cancer and pancreatic cancer.

Table 1-3. Summary of the application of CSLPHNPs in drug delivery

Encapsulant	Polymer	Lipid	Particle size	EE/DL	Application	Reference
siRNA and Gemcitabine	mPEG-PLGA	Lecithin,cholesterol, DSPE-PEG2000	122-142 nm	EE 42 %	Pancreatic cancer	(190)
Curcumin and RGD	mPEG-PLGA	Lecithin, cholesterol, RGD-PEG-Chol	216 nm	EE 96 % DL 5 %	Brain targeting for glioma	(70)
Mitomycin C	PLA	SPC	200-300 nm	EE 37 % DL 10 %	Cancer	(191)
Lysozyme	PCL	PC, glyceryl tripalmitate	90-470 nm	EE 45-90%	Protein delivery	(192)
siRNA	PLGA	DOTAP	213-286 nm	EE 30 %	Gene delivery	(193)
Doxorubicin and GG918	HPESO	Tristearin/ stearic acid	150-270 nm	EE 70–90 %	MDR breast cancer	(99)
Doxorubicin	HPESO	Stearic acid	290 nm	EE 76 %	MDR breast cancer	(100)
Doxorubicin	PLGA	DPPC	195 nm	DL 1 %	MDR breast cancer	(138)
Paclitaxel	PLGA	Lecithin	83-95 nm	NR	Pancreatic cancer	(94)
Verapamil HCl	Dextran	Decanoic acid	342 nm	EE 90-99 %	NR	(96)
Paclitaxel	PLGA	DLPC	200-300 nm	EE 43-56 %	Cancer	(98)
Paclitaxel	PLGA	OQLCS	184-194 nm	EE 84-88 %	Cancer	(135)
Docetaxel, indium 111 and yttrium 90	PLGA	Docetaxel, indium 111 and yttrium 90	65 nm	EE 60 %	Prostate cancer	(134)

Table 1-3. Continued

Encapsulant	Polymer	Lipid	Particle size	EE/DL	Application	Reference
AChE	PMOXA- PDMS- PMOXA	EPC/DPPC	75 nm	NR	Protein delivery	(91)
Docetaxel	PLGA	Soy lecithin	60-70 nm	NR	Cancer	(86)
Docetaxel	PLGA	Lecithin/DSPE-PEG	70-80 nm	EE 60 %	Cancer	(69)
Plasmid DNA	PEI	Triolein/EPC/ DSPE-PEG	128 nm	NR	Gene delivery	(95)
Plasmid DNA	PLGA	DOTAP/DC-Chol	100-400 nm	NR	Gene delivery	(154)
Plasmid DNA	PLA	DPPC/DPTAP	325-340 nm	NR	Gene delivery	(103)
mRNA	PBAE	DOPC/DOTAP	230-300 nm	NR	mRNA based vaccine	(194)
siRNA	PLGA	EPC/lecithin/DSPE- PEG	225 nm	NR	Tumor suppression	(124)
7 α -APTADD	PLGA	Egg PC/DOPE/ TPGS	170 nm	EE 78-82 %	Breast cancer	(136)
Fluoroquinolone antibiotics	PLGA	Phosphatidyl choline	260-420 nm	EE 37 %	Lung biofilm infection	(141)
5-Fluorouracil	PGA/ Dextran	Cetyl alcohol /Tripalmitin	600-1100 nm	EE 4-25 %	Lung cancer	(130-132)
FITC-BSA	Protamine sulfate	Cholesterol/ DSPC/DHA	130-200 nm	EE 19-60 % DL 5-18 %	Protein delivery	(104)

Vaccine adjuvants. NPs are promising adjuvant delivery systems for enhancing and directing the adaptive immune response of vaccine antigens (189). Biodegradable polymeric microparticles and NPs composed of PLGA have been investigated as potential vaccine delivery systems due to their ability to control the release of antigens and co-delivering immunostimulatory molecules along with antigens in the same particle (195). However, low antigen EE and denaturation of the antigen during nanoencapsulation have limited their development (196, 197). Antigens adsorbed or covalently coupled onto the surface of pre-synthesized CSLPHNPs could be viable strategies for vaccine delivery (105, 198). “Synthetic pathogens”, which are surface-modified biodegradable CSLPHNPs, can be used to imitate structural features of pathogens for designing vaccine adjuvants (137). In this context, polymeric core nanoparticles (e.g. PLGA) are advantageous since they activate inflammasome in antigen-presenting cells and enhance innate/adaptive immune responses (199). In addition, lipid bilayers displaying protein antigens and molecular “danger signals” (such as pathogen-associated molecular patterns) create pathogen-mimicking antigens and related motifs to boost the immune response (105). The surface display of antigen onto lipid-based NPs has been shown to induce robust antibody responses by mimicking the structure and surface chemistry of microbial pathogens (200). For example, high IgG titers ($> 10^6$) were observed with sustained levels over 100 days after immunization with nanograms of ovalbumin antigen conjugated onto the surface of CSLPHNPs along with monophosphoryl lipid A or α -galactosylceramide as molecular “danger signals” (105). Moreover, the strategy allows the conjugation reaction to proceed under mild aqueous conditions thus avoiding harsh processing during encapsulation (178). Additionally, the immune response can be altered by the presence of heterogeneous surface functional groups. It has been reported that the presence of amine terminal group of DSPE-PEG on the PLGA-lecithin CSLPHNPs induced highest complement activation and could be considered as vaccine adjuvants (153).

Cancer targeting. Recent advancements in nanotechnology have fuelled NPs development of different sizes, shapes, core physicochemical properties, and surface modifications for the potential treatment of cancers. CSLPHNPs are being developed for tumor-selective delivery of anticancer agents to increase the cell-kill effect while protecting the healthy tissue from exposure to a cytotoxic agents, thereby reducing systemic toxic effects (142). The following section discusses selected studies dealing with *in vitro* evaluation and *in vivo* evaluation. Most of the literature on CSLPHNPs has focused on *in vitro* cell culture models as the means to proof of concept.

Breast cancer is the most common form of cancer and affected more than 200,000 females in 2010 in the United States. Multidrug resistance (MDR) is a common cause of failure of chemotherapy in breast cancer patients (201). MDR is caused by overexpression of membrane drug efflux transporter P-glycoprotein (P-gp), which reduces intracellular uptake of anticancer drugs (202). Excellent reviews are available on the cause and strategies for overcoming MDR (203, 204). For example, a CSLPHNPs system containing doxorubicin was developed and evaluated for cytotoxicity against MDR breast cancer cells by Wong *et al* (99-102). The particle size and EE of the

CSLPHNPs were reported to be 50-200 nm and 65-80 %, respectively. Cell-kill and cellular uptake were significantly enhanced in CSLPHNPs forms compared to the solution formulation. Two possibilities for the mechanism of cytotoxicity of doxorubicin-loaded CSLPHNPs were proposed: i) free drug was released from CSLPHNPs and acted on the cells, and ii) drug-loaded CSLPHNPs entered and released the drug inside cells, thereby evading the P-gp efflux pump. In a subsequent publication, the authors proposed that the second mechanism was more likely to happen. Drugs in CSLPHNPs entered the cells by a combination of diffusion and phagocytosis. Because of the physical association of the drug with the anionic polymer, the drug was not easily removed by the P-gp efflux pump. Therefore, chronic suppression of the MDR cell proliferation was observed because of the continued buildup of drug inside cells (98).

Another potential strategy to overcome MDR of breast cancer cells is to simultaneously use a combination of chemotherapeutic drug and P-gp inhibitor /chemosensitizer such as verapamil in a single nanoparticle cargo (204). Similar strategy using a CSLPHNPs capable of co-delivering doxorubicin and elacrider (chemosensitizer) was developed and evaluated by Wong *et al* (99). The particle size was found to be 187-272 nm. EE was 71-76 % for doxorubicin and 80-88 % for elacrider. In this study, the dual agents co-encapsulated in CSLPHNPs showed greatest uptake and anticancer activity in human MDR breast cancer cell line MDA435/LCC6/MDR.

In another study, a CSLPHNPs system having PLGA core and phosphatidyl choline shell was designed for loading 7 α -APTADD, an investigational aromatase inhibitor for treatment of estrogen-responsive breast cancer (136). Transferrin, a natural 80 kDa glycoprotein, was conjugated to CSLPHN to target SKBR-3 breast cancer cells with overexpressed transferrin receptors. EE and mean diameter were measured to be 37 % and 170 nm. Aromatase inhibition activity of the targeted CSLPHNPs was significantly higher in SKBR-3 cells compared to non-targeted CSLPHNPs.

Prostate cancer is the second leading cause of cancer mortality in men over the age of 40 in the United States (205). Prostate-specific membrane antigen (PSMA) is a type II membrane integral glycoprotein overexpressed in prostate cancer cells and has been identified as a biochemical marker (206). Several PNPs and liposomal targeted delivery systems were developed for prostate cancer (207-209). A new CSLPHNPs system composed of a PLGA core and lecithin/DMPE-DTPA lipid shell was developed for prostate cancer by co-delivering chemotherapeutic drug docetaxel (Dtxl) and the therapeutic radionuclide yttrium 90 (⁹⁰Y) (134). The CSLPHNPs were termed as “chemorad NPs”. They were prepared by the single-step nanoprecipitation method to produce mean particle size of 65 nm. Oligonucleotide aptamer A10, which has high affinity and selectivity to PSMA positive prostate cancer cells, was attached to the outer surface of the CSLPHNPs *via* coupling reaction with DSPE-PEG to produce targeted particles (Apt-Dtxl-⁹⁰Y-NPs). An increase in uptake of chemorad NPs was observed in the LNCaP prostate cancer cell lines. “Chemorad NPs” were able to kill 80 % of the LNCaP cells (PSMA-positive) compared to the PC3 cell line (PSMA-negative) and untargeted control groups. The experimental findings of this study suggested the potential of chemorad NPs to improve chemoradiotherapy in prostate cancer patients.

Pancreatic cancer, especially adenocarcinoma of the exocrine pancreas, is the fourth leading cause of cancer death in the United States (210). However, the current chemotherapeutic regimen showed little or modest improvement in patient survival because of poor vascularization and inadequate perfusion of the tumor (211). Liposomal paclitaxel and gemcitabine (212), cisplatin and gemcitabine (213), curcumin-encapsulated PLGA NPs (214), and EGFR-targeted gemcitabine-loaded PLGA NPs (215) showed promising efficacy in refractory pancreatic cancer in animal studies and clinical trials. Anti-carcinoembryonic antigen (CEA) half- antibody was conjugated to paclitaxel-loaded CSLPHNPs, which were investigated for targeting ability against BxPC-3 (CEA-positive) and XPA-3 (CEA-negative) pancreatic cancer cells (94). Antibody conjugated CSLPHNPs with particle size of 95 nm were prepared by nanoprecipitation via self-assembly of PLGA, lecithin, and DSPE-PEG. Monoclonal antibody was attached to CSLPHNPs through a maleimide-thiol coupling reaction. Targeting specificity, as well as enhanced cellular cytotoxicity of paclitaxel-loaded CSLPHNPs was observed in CEA positive cells compared to their non-targeted counterparts. This can be explained by occurrence of the receptor mediated endocytosis process which facilitated particle internalization into cells. Thus, the delivery platform showed therapeutic potential of CSLPHNPs in the targeting of pancreatic cancer.

There are few available studies reported in the literature based on *in vivo* evaluation of drug loaded CSLPHNPs in animal cancer models (101, 131-133, 135). To best of my knowledge, the pioneering research involving *in vivo* evaluation of dual drug loaded CSLPHNPs, known as “nanocell”, was reported in 2005 by Sengupta *et al* (133). The delivery system comprised of chemotherapeutic agent doxorubicin conjugated to PLGA forming polymeric core (nucleus, similar to a cell) and the antiangiogenic agent combretastatin entrapped within the lipid shell. Tumors were induced by implanting GFP-positive BL6/F10 melanoma cells or lewis lung carcinoma cells in male c57/BL6 mice. Intravenous administration of different combinations of CSLPHNPs containing doxorubicin and/ combretastatin showed that CSLPHNPs containing dual agents exhibited distinctly greater reduction in tumor volume with increasing percent survival in Kaplan-Meier survival graphs compared to CSLPHNPs with other combinations. The study proved that the dual agent loaded-CSLPHNPs treatment induced inhibition of tumor growth in a dose-dependent manner with more susceptibility towards melanoma than lung carcinoma. In addition, white blood cell count assay indicated that the delivery system resulted in least systemic toxicity compared with other combinations.

Another study dealt with the *in vivo* evaluation of the doxorubicin-loaded CSLPHNPs in the solid tumor model induced by injecting EMT6 mouse mammary cancer cells intramuscularly into the hind legs of BALB/c mice (101). The cationic anticancer agent doxorubicin was complexed with anionic polymer HPESO to form a core, which was then covered by the lipid mixture of stearic acid and tristearin. The mean time for the tumor to reach the cutoff size was significantly prolonged by 7 days. The tumor growth delay value was 100% in mice after receiving 0.2 mg of doxorubicin in the form of CSLPHNPs compared to blank CSLPHNPs injected into the tumor. The normal tissue toxicity of the particles was minimal after a single dose of intratumoral injection, suggesting the usefulness of the delivery system for local treatment of breast cancer.

Gene delivery. Delivery of nucleic acids represents a challenge and great opportunities to treat chronic diseases, genetic disorders, and cancers (216). Cationic liposomes and biodegradable PNPs have been investigated as gene delivery carriers (217). Polymer-based non-viral carriers have received significant attention because of the death of a patient in a clinical trial of viral-based gene therapy (218). Among various non-viral based approaches, particularly polymer and lipid-based non-viral carriers have several advantages: low immunogenicity, low toxicity, absence of viral recombination, low production cost, and the possibility of repeated administration (219).

Cytotoxicity, stability in serum, duration of gene expression, and particle size of the non-viral based carriers still remain major limitations of lipid and polymer-based systems. Recently, CSLPHNPs have emerged as an alternative, biodegradable, stable, and long-lived nanoparticle vector delivery system. Plasmid DNA encoding luciferase reporter gene was entrapped in CSLPHNPs composed of PLGA and cationic lipids DOTAP/DC-cholesterol (154). The CSLPHNPs (100-400 nm) were able to transfect the luciferase gene in adherent 293 human prostate cancer cells 500-600 times more efficiently than did unbound DNA after 48 h. Another CSLPHN was reported by Li *et al* (95) for efficient non-viral gene delivery with higher transfection efficiency and lower toxicity compared to commercial LipofectamineTM2000. In another study, CSLPHNPs with a mean particle size of 128 nm were prepared by emulsion evaporation technique using different combinations of triolein, polyethylenimine (PEI), egg yolk phosphatidylcholine (EPC), and PEG-DSPE. Plasmid DNA was complexed with NPs by adsorption. A green fluorescent protein intensity study revealed that the transfection efficiencies of CSLPHNPs/DNA complexes were 37 % and 34 % for HEK293 and MDA-MB-231 cells, respectively. Transfection efficiency was significantly higher than that of commercial LipofectamineTM 2000. Additionally, the proton-sponge effect destabilized the endosomal membrane and enhanced transfection. PEG helped as a protective layer and reduced the degradation of plasmid DNA by lysosomal enzymes after entering the lysosome.

For siRNA delivery, cationic nanoscale complexes such as lipoplexes or polyplexes were used successfully to deliver siRNA (220). But some of these systems have disadvantages such as toxicity, induction of inflammatory responses and instability in serum. Shi and coworkers (124) designed a relatively neutral surface charged hybrid nanostructure capable of protecting siRNA and lipoplexes from physiological environments. This delivery system was termed “differentially charged hollow core/shell lipid-polymer-lipid hybrid nanoparticles” which were composed of four functional building blocks; a positively charged inner hollow core made up of cationic lipid, a hydrophobic PLGA layer, with a neutral lipid layer having outer PEG chains. Combination of a modified double emulsion solvent evaporation method and a self-assemble method yielded an average particle size of around 225 nm and neutral surface charge. The hybrid system was capable of releasing siRNA in a sustained manner, enhanced *in vivo* gene silencing and inhibited luciferase gene expression in murine xenograft tumors. This strategy has opened another potential avenue for successful gene delivery for the treatment of multidrug resistant cancers.

Summary, future prospects and challenges of CSLPHNPs

CSLPHNPs are the alternative platform for drug delivery. These particles design uses an integrative approach by combining two classes of nanocarriers, namely polymeric NPs and liposomes. These particles have several beneficial features for treating various diseases, particularly cancers. Often treatment of a single type of cancer requires administering multiple drugs, and, in this aspect, CSLPHNPs are promising because they have to the potential to deliver multiple drugs simultaneously from a single platform. Specifically, incorporating two drugs into the core and lipid layer can offer a viable approach to treat MDR and life-threatening diseases. Apart from small molecular weight drugs, delivery of diagnostic agents such as quantum dots, macromolecules such as proteins, and genes, offers other exciting strategies with CSLPHNPs. Because their structural similarity to the viral architecture, CSLPHNPs offer potential as vaccine adjuvants. Furthermore, recent advancements in the CSLPHNPs delivery system such as coating PNP with natural erythrocyte membrane, entrapping quantum dots inside these hybrid particles and concurrent administration of chemotherapy/radiotherapy, have shown potential for theranostic applications in the treatment of malignancies and other diseases.

The design and development of CSLPHNPs as drug delivery platforms have been concentrated in the architecture and *in vitro* efficacy. The complexities of these systems afford new challenges in translating the *in vitro* efficacies into tangible therapeutic options. More focused research is warranted, especially in key areas of development including stability, scale-up, optimization of targeting ligand density, *in vivo* fate, toxicity, and pharmacokinetic profiles.

Stability of new drug product is an essential prerequisite. Therefore, the long-term physical and chemical stability of these hybrid nanoparticles in various environmental stress conditions need to be systematically evaluated to have a shelf life assigned to the marketed product. The critical parameters that should be evaluated include, but are not limited to, particle size distribution, drug entrapment, retention of entrapped drug in the system, physical robustness of the system, and effect of stressed environments on any of the aforementioned parameters (221). As with any colloidal system, stability can be a challenge in the liquid state. Thus, if instability is observed in aqueous states, other strategies could be evaluated including lyophilization or other stabilization techniques to address instability issues (170).

Active targeting has been considered to be a significant shift in the paradigm of therapeutic efficacy of nanoparticulate drug delivery systems (41). Although, these systems show potential in early *in vitro* or “proof-of-concept” studies, a number of factors that can impact its efficacy need to be addressed. One such factor is the optimization of the targeting ligand on the hybrid NPs surface. The selection of the targeting ligands should additionally be evaluated because some of these targeting agents possess pharmacological activity (222). Understanding of the targeting ligand is crucial to address therapeutic outcomes and also address confounding outcomes due to polypharmacological inconsistencies.

The pharmacokinetic and pharmacodynamics (PK/PD) effects of these systems should be critically evaluated. Traditional PK evaluations are dependent of the availability of the free drug in the biological system to postulate its PD or metabolic fate. When drugs encapsulated in CSLPHNPs are administered, the PK/PD profile may be altered when compared to only the free drug due to altered release of the drug from these systems. Therefore, the appreciation of this phenomenon is essential in understanding the final therapeutic outcomes of these systems. A recent review by Li and Huang specifically addresses this aspect of the PK/PD fate of NPs and should be a valuable resource for the readers (46).

A primary requirement for any product entering the pharmaceutical market is the availability of large scale production methods which need to be cost-effective and meet the regulatory requirements. Current bench scale processes employed for the developing CSLPHNPs systems are labor intensive and are not amenable to direct scale-up. Moreover, most of these delivery systems are intended for parenteral administration and thus directly impact its aseptic production. Although, significant advances in aseptic processing have been utilized for the manufacturing of CSLPHNPs systems, it often comes with a high price-tag and can be cost-prohibitive.

Central Hypothesis and Specific Aims

Lung cancer is the leading cause of cancer related death for both men and women worldwide (1). NSCLC represents 85 to 90 % of all lung cancer cases. Lung cancer patients are diagnosed at an advanced inoperable stage of the disease with the main treatment option being chemotherapy with cytotoxic drugs. However, the 5-year survival rate at advanced stages (III and IV) of NSCLC remains only about 15 % and 1 % respectively (4). One of the main reasons for the poor survival rates among patients with advanced stages of NSCLC is the limited efficacy of traditional chemotherapy along with severe adverse effects of the high doses of cytotoxic anticancer drugs. Besides cytotoxic chemotherapies, a number of molecularly targeted agents were developed exploiting two major cancer cell pathways: EGFR and VEGFR (the vascular endothelial growth factor receptor). EGFR has been identified as the effective molecular target and overexpressed (50-90 %) in several cancer types including NSCLC (5). In 2004, US FDA approved the drug, erlotinib (ETB) for the treatment of locally advanced or metastatic NSCLC after failure of at least one chemotherapy regimen. ETB is a highly selective inhibitor of HER1/EGFR-TK. The drug binds with the ATP binding site of the tyrosine kinase domain of EGFR, which blocks the catalytic activity of the kinase, thereby inhibiting downstream signaling of the pathways responsible for cell proliferation, cell survival, angiogenesis, and metastasis (6). However, limited therapeutic efficacy along with several toxicities was reported from the traditional oral delivery of ETB (7, 8). Additionally, administration of oral ETB tablets is not convenient to cancer patients with gastrointestinal disorders/abnormalities (27). Another major concern with ETB therapy is the development of drug resistance by P-glycoprotein mediated efflux pump or other pathways in NSCLC tumors (30).

The low survival rate in NSCLC patients demands the development of novel and efficacious therapeutic approaches for the treatment of NSCLC. Nanoparticulate carriers are attractive for cancer therapy due to their ability to enhance accumulation of anticancer agents at the tumor site, resulting in increased tumor cell kill efficacy, reduction in dose and dose-related adverse effects to healthy cells (41). There has been some investigations on the ETB loaded NPs reported in the literature. ETB loaded polymeric NPs in mice has shown improved efficacy and reduced toxicities associated with the drug (223). Another study revealed polymeric NPs improved antitumor efficacy of ETB in A549 human lung adenocarcinoma cells (224). Formulation of reverse micelles containing ETB showed improved *in vitro* efficacy of ETB in pancreatic cancer cells (225). Huang and coworkers examined the inhibition of HER1/EGFR-TK signaling using an anti-EGFR monoclonal antibody (mAb) and tyrosine kinase inhibitor, which target extracellular and intracellular domains of the receptor (14). This approach suggests potential new strategy to maximize effective target inhibition, which may improve the therapeutic efficacy for the treatment of NSCLC. Recently, our group has published a review on core-shell-type lipid-polymer hybrid nanoparticles as drug delivery platform (65). This unique platform combines the mechanical advantages of biodegradable polymeric nanoparticles and biomimetic advantages of liposomes into a single, integrated platform. The system provides advantages such as controllable particle size, surface functionality, high drug loading, potential for entrapment of multiple therapeutic agents, tunable drug release profile, and good serum stability (65). For the proposed study, core-shell type lipid-albumin hybrid nanoparticles (CSLAHNPs) were utilized as a novel and unique nanoparticulate carrier for the delivery of ETB in NSCLC. The schematic diagram of the proposed delivery system was shown in **Figure 1-6**. The core was composed of albumin which is a natural protein present in blood. The shell were composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) which is a major lung surfactant phospholipid. Both albumin and phospholipid are biocompatible and biodegradable. Finally, anti-EGFR monoclonal half-antibody (hAb) were conjugated to the CSLAHNPs.

The central hypothesis of my project is that permeability and uptake of anti-EGFR half-antibody (hAb) conjugated erlotinib (ETB)-loaded CSLAHNPs (targeted) and erlotinib (ETB)-loaded CSLAHNPs in NSCLC tumor cells is expected to improve the therapeutic efficacy of ETB while reducing off-toxicities of the drug in the treatment of EGFR positive NSCLC compared to free ETB in solution (**Figure 1-7**). Therefore, in order to test the hypothesis, the following specific aims were proposed:

Aim 1. To design, develop, characterize and optimize CSLAHNPs

The purpose of this specific aim was to prepare and characterize blank CSLAHNPs without encapsulation of drug ETB to understand the CSLAHNPs delivery platform. Formulation and process parameters that influence physicochemical properties including mean size, polydispersity index (PDI), zeta potential, morphology, and related critical quality attributes CSLAHNPs were identified and optimized to guide subsequent design, preparation and optimization of ETB-loaded CSLAHNPs.

Figure 1-7. Schematic representation of the central hypothesis

Targeted anti-EGFR half-antibody conjugated ETB-loaded CSLAHNPs bind to the extracellular domain of EGFR overexpressed on human NSCLC cells. After receptor binding and internalization, the drug is released in the cytoplasm and binds with intracellular tyrosine kinase domain of EGFR and prevents subsequent downstream signaling pathways.

Aim 2. To develop, characterize and optimize erlotinib loaded CSLAHNPs

The purpose of this specific aim was to prepare and characterize erlotinib loaded CSLAHNPs (ETB-CSLAHNPs). Various strategies were used for drug loading in CSLAHNPs. Formulation and process parameters that influence mean size, PDI, zeta potential, drug loading, drug entrapment efficiency, morphology, drug release, storage and serum stability CSLAHNPs were identified and optimized.

Aim 3. To develop, characterize and optimize targeted erlotinib-loaded CSLAHNPs

Anti-EGFR monoclonal antibody (mAb) was selectively reduced to half-antibody (hAb) fragments and conjugated to ETB-CSLAHNPs via maleimide-thiol conjugation for the preparation of targeted anti-EGFR hAb conjugated erlotinib-loaded CSLAHNPs (hAb-ETB-CSLAHNPs). The hAb-ETB-CSLAHNPs were characterized for mean size, PDI, zeta potential, drug loading, drug entrapment efficiency, morphology, drug release, storage and serum stability CSLAHNPs.

Aim 4. To evaluate cellular uptake, and efficacy of CSLAHNPs in human NSCLC cells

The cellular uptake, efficacy, mechanism of uptake, intracellular trafficking, and up/downregulation of p-EGFR after the treatment of the targeted and untargeted ETB-CSLAHNPs were evaluated in human lung cancer cells (A549 and HCC827).

Aim 5. To lyophilize and develop stable CSLAHNPs

The purpose of this specific aim was to improve the shelf-life of the CSLAHNPs *via* lyophilization process. The untargeted and targeted liquid formulations were subjected to lyophilization process to preserve CSLAHNPs as dry powder. The stability of liquid dispersion and lyophilized dry powder were evaluated after stored at different storage conditions for various lengths of time.

CHAPTER 2. PREPARATION, CHARACTERIZATION, AND OPTIMIZATION OF CORE SHELL TYPE LIPID ALBUMIN HYBRID NANOPARTICLES

Introduction

Protein based colloidal drug delivery systems prepared from plasma proteins have attracted much attention because they offer several advantages: biodegradability, biocompatibility, non-antigenic, easy to prepare, controlled particle size distribution, various possibilities for surface modification, greater stability, relatively easy to scale up and binding ability for a number of hydrophilic and lipophilic drug molecules (226, 227). Among various plasma proteins, albumin has been widely used in versatile drug delivery systems including microparticles, nanoparticles (NPs), and micelles for various drugs and diagnostic agents (226, 228). Three different types of albumins can be used in drug delivery purposes: ovalbumin (229), bovine serum albumin (230), and human serum albumin (231). Significant amount of drug can be loaded into albumin matrix due to the presence of drug binding sites (232). Additionally, presence of high quantity of charged amino acids allow electrostatic attraction and loading of charged drug molecules and oligonucleotides into albumin matrix (233-236). A significant advantage of albumin NPs includes its accumulation to the tumor and inflamed site. This is due to the passive targeting or enhanced permeation and retention (EPR) effect and its active targeting ability mediated by binding with endothelial gp60 receptors overexpressed on these tissues (237, 238). Marketed products include albumin NP bound paclitaxel (AbraxaneTM) which was approved by the US Food and Drug Administration in 2006 for the treatment of metastatic breast cancer (237, 239, 240). For diagnostic applications, two albumin NPs based products (Nanocoll[®] and Albures[®]) were approved for the detection of cancer and rheumatoid arthritis respectively (228). Among different types of albumin, bovine serum albumin (BSA) has been widely used to prepare drug delivery systems including NPs because of its defined primary structure, non-antigenicity (241), stability, abundance, low cost, ease of purification, and ligand binding properties (226). It is a globular protein with molecular weight of ~ 66 kDa and isoelectric point (pI) of 4.7 in water at 25°C (230, 242). It has a single polypeptide chain consisting of about 583 amino acids residues, 17 intrachain disulfide bridges, and 1 sulfhydryl group (243). BSA based albumin NPs (ANPs) are produced by desolvation or coacervation (244-248), emulsification (249, 250), thermal gelation (251, 252), nanospray drying (253), nab-technology (254), disulfide bond reduction (255, 256), and self-assembly methods (257). Desolvation is the most widely used method for the preparation of ANPs. Detailed investigations on the formulation and process variables of the desolvation method were reported in the literature (244-246, 258).

NPs can broadly be classified as simple NPs and composite or core-shell NPs based on the number of materials used and the structural features (259). Simple NPs as the name implies are composed of a single material whereas composite NPs are made up of two or more materials. The focus of nanoparticle design over the years has evolved towards more complex nanoscopic core-shell architecture to combine multiple

functionalities (116, 260, 261). Core-shell NPs can broadly be described as NPs comprising of a core (inner material) and shell (outer layer material) (259). A wide range of combinations of inner and outer materials were used such as organic/organic, organic/inorganic, inorganic/organic, and inorganic/inorganic (259). Significant advancement in nanotechnology has enabled to produce not only core-shell NPs with spherical or symmetric shapes but also other non-spherical shapes (259).

Core-shell type lipid-polymer hybrid nanoparticles (CSLPHNPs), which combine the mechanical advantages of biodegradable polymeric NPs and biomimetic advantages of liposomes, have emerged as a robust and promising delivery platform (65, 82, 262). In CSLPHNPs, a biodegradable polymeric core is surrounded by a shell composed of layer(s) of phospholipids. Various bioactive molecules such as drugs, genes, proteins, and targeting ligands can be entrapped, adsorbed, or covalently attached in the hybrid system. The common choices of biodegradable polymers include polylactic-*co*-glycolic acid (PLGA), polycaprolactone (PCL), dextran due to their biocompatibility, biodegradability, non-toxicity and previous use in approved products (83, 84). Lipids used are often zwitterionic, cationic, anionic and neutral phospholipids. The examples include lecithin, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) or 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). The hybrid architecture can provide advantages such as controllable particle size, surface functionality, high drug loading, entrapment of multiple therapeutic agents, tunable drug release profile, and good serum stability (134). Besides passive targeting of CSLPHNPs based on particle size, they can be conjugated with appropriate targeting ligands such as aptamers (134), folic acid (97, 135), transferrin (136), antibody (94), and single-chain tumor necrosis factor (121) to deliver NPs at the target tissues for treating cancers.

In this project, a unique drug delivery system known as core-shell type lipid albumin hybrid nanoparticles (CSLAHNPs) was proposed. The delivery system was comprised of albumin core surrounded by phospholipid layer for the delivery of ETB, a small-molecule EGFR tyrosine kinase inhibitor for the therapy of NSCLC (previously described in Chapter 1. Core-shell type hybrid NPs comprising of albumin core is a unique strategy which has not been reported in the literature till date. BSA was selected as the biodegradable polymer to: i) form the core of the CSLPHNPs, ii) to support the phospholipid layer, and iii) to bind the hydrophobic drug molecules. The shell of CSLAHNPs was composed of DPPC, cholesterol, and DSPE-PEG2000 with a molar ratio of 60:30:10 respectively. DPPC is the major lung surfactant phospholipid having a polar head group and two non-polar hydrocarbon tails of palmitic acid (263). Incorporation of DSPE-PEG2000 has become a preferred strategy to reduce NPs uptake by RES organs and to increase NPs circulation time (264). All the components (BSA, DPPC and DSPE-PEG2000) have been approved by the Food and Drug Administration (FDA) for medical applications. CSLAHNPs were expected to be biocompatible, biodegradable, and potentially safe as a drug carrier for clinical use.

Particle size and surface characteristics are the two important physicochemical properties of any nanoparticle based delivery system for intravenous administration.

These properties mainly affect their transport across biological membranes, body distribution, and cellular uptake (265-267). The functional or physiologic pore size of the renal glomerular capillary was found to be 4.5-5 nm in diameter (268). Hepatobiliary clearance eliminates viruses and other small particles of size 10-20 nm (269). In addition to size, surface characteristics of NPs influence opsonization process by which proteins are adsorbed on the particle surface and leads to elimination of the particles from systemic circulation (264). The mean diameter of blood capillaries or microvessels which connect arterioles and venules was found to be around 5-10 μm (270). Uncoated bare NPs of size in the range 2-300 nm are taken up by the liver sinusoidal endothelial cells while greater than 2-300 nm sized particles are taken up by the Kupffer cells. Therefore, prolonged circulation of NPs in the blood is desired because it would increase the probability of extravasation of NPs into the target tissues. For intravenously injected NPs, a size range of 100-300 nm with a hydrophilic particle surface is expected to be optimum for drug delivery applications. Majority of the marketed therapeutic and diagnostic NP-based products falls into the size range of 100-300nm. Apart from size, the size distribution of NPs is another important parameter. It is measured by a parameter called polydispersity index (PDI). Conceptually, PDI is the measure of the width of particle size distribution and expressed by a dimensionless number between 0 to 1 (271). If PDI value is lesser than 0.1, it is considered monodispersed size distribution (272). For the present study, PDI below 0.2 was considered acceptable. Another important NP property is the surface charge which is measured by zeta potential. The electrostatic potential at the electrical double layer surrounding the NPs in solution is known as zeta potential. NPs with zeta potential close to ± 10 mV is considered neutral whereas zeta potential greater than ± 30 mV indicates strong electropositivity or electronegativity (273). NPs with near neutral zeta potential or mildly charged surfaces tend to aggregate faster, which may enhance clearance by the RES. Stronger the surface charge, better is the colloidal stability of NPs and hence a longer shelf life on storage as well (274). Cationic NPs was found to interact more with the negatively charged cell membranes thereby enhancing toxicity to healthy tissues whereas anionic NPs was found to be non-toxic (275). NPs with negative zeta potential with a magnitude lesser than -30 mV are desirable for *in vivo* stability and also for colloidal stability on storage. Therefore, optimization of formulation and process parameters which control the particle size and surface characteristics would be a rational approach to improve the efficacy of NPs in biological system. Therefore, the objective of this study was to identify and optimize formulation and process parameters, and to develop a prototype formulation to prepare blank CSLAHNPs which could be utilized subsequently for drug loading and antibody conjugation.

Experimental Section

Materials

Bovine serum albumin (BSA, Cohn fraction V, lyophilized powder, MW~66000, purity 95-99%) was obtained from Sigma-Aldrich (St. Louis, MO). The lipids used in this work were all of research grade. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)

and 1,2-distearoyl-sn-glycero-3-hoshoethanolamine-N (poly(ethylene glycol)2000) [DSPE-PEG2000] were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol, ferric chloride hexahydrate, and ammonium thiocyanate were purchased from Sigma (St. Louis, MO). High performance liquid chromatography (HPLC) grade water, ethanol, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). HPLC grade chloroform and 1N sodium hydroxide solution were purchased from Acros Organics (Morris Plains, NJ). All other chemicals and reagents were of analytical grade and used without further purification or characterization.

Preparation of CSLAHNPs

CSLAHNPs were prepared using a two-step method which combined desolvation method (245) and lipid film hydration method (133, 159). The schematic representation of the two-step method was shown in **Figure 2-1**.

In the first step, albumin nanoparticles (ANPs) were prepared by the desolvation (also known as pH-coacervation) method widely reported (244, 258, 276-278). Briefly, BSA was dissolved in 10 mM sodium chloride solution and pH was adjusted to 6- 9 with 1N sodium hydroxide solution. Ethanol was added dropwise at 0.5-2 mL/min into the aqueous phase (water/ethanol volume ratio 1:2) until turbidity appeared. The coacervates formed were hardened for 12 h after adding glutaraldehyde solution and the resulting albumin nanoparticles were purified by centrifugation at 12000 rpm for 30 min, washed 3 times and reconstituted in 1x PBS.

In the second step, lipids (DPPC, cholesterol, DSPE-PEG2000; molar ratio 60:30:10) dissolved in methanol: ethanol (50:50 v/v) was evaporated in vacuum evaporator in a round-bottom flask. The dry lipid film was hydrated with ANPs dispersion (1x PBS) for 15-30 min at 45-50°C. After hydration, two separate methods were utilized for CSLAHNPs preparation; sonication and extrusion. The dispersion was sonicated in water bath for 10 min. In parallel, another batch was hydrated with ANPs dispersion (1x PBS) for 15-30 min at 45-50°C and extruded through a stack of polycarbonate membranes (0.8 µm/0.4 µm/ 0.2 µm) using mini-extruder. The free vesicles and micelles were removed by ultracentrifugation at 10000-14000 rpm for 20-30 min, followed by washing thrice. The unbound free drug and other small molecular weight species were removed by gel filtration column (sepharose CL 4B) by spinning the sample at 400 g for 5 min with 1x PBS as the elution buffer. The final product was reconstituted in 1x PBS.

Optimization of ANPs

A number of formulation and process variables were selected to produce optimum core ANPs with size (100-250 nm), PDI lesser than 0.2 and zeta potential lesser than -30 mV. These variables included the pH of albumin solution (6-9), albumin concentration

Figure 2-1. Schematic representation of the two-step method for the preparation of blank CSLAHNPs

In the step I, blank core ANPs were prepared by adding ethanol dropwise, followed by hardening with glutaraldehyde. In step II, mixture of lipids (DPPC, cholesterol, DSPE-PEG2000 at a molar ratio of 60:30:10) were dissolved in ethanol:methanol (50:50 v/v), followed by evaporation of organic solvents to form a dry lipid film. Finally, the film was hydrated with ANPs to form blank CSLAHNPs.

(5-40 mg/mL), rate of addition of ethanol (0.5-2 mL/min), concentration of the cross-linker glutaraldehyde (0.188-0.97 % w/w), and water-to-ethanol volume ratio (1:1-1:4).

Optimization of CSLAHNPs

Since preparation of CSLAHNPs required incubation of ANPs at or above the transition temperature of lipids, the thermal stability of ANPs was determined. Briefly, ANPs were incubated at 45-50°C for 30 min, followed by determination of the physicochemical properties (size, PDI, and zeta potential). The best method to prepare CSLAHNPs was selected; either sonication or extrusion. The most important factor that influenced the physicochemical properties of CSLAHNPs was total lipid-to-albumin ratio ($W_{\text{Lipid}}/W_{\text{ANPs}}$). An empirical calculation was performed to predict the $W_{\text{Lipid}}/W_{\text{ANPs}}$ based on the assumptions; the spherical ANPs, 100 % DPPC in the bilayer, 5 nm lipid bilayer thickness. In parallel, different $W_{\text{Lipid}}/W_{\text{ANPs}}$ ratios were selected (0, 5, 10, 15, 20, and 30 %) to prepare blank CSLAHNPs.

Characterization of ANPs and CSLAHNPs

The mean hydrodynamic size (Z-average) of ANPs and CSLAHNPs was determined by dynamic light scattering (also known as photon correlation spectroscopy) principle using Zetasizer Nano ZS90 (Malvern Instruments, Westborough, MA) equipped with 50 mW diode laser as a source of light operating at 532/633 nm. Particle scattered photons were detected at an angle of 90°. The samples were suitably diluted with HPLC grade water for the determination of Z-avg. Three independent measurements were performed for each sample. Zeta potential was determined by electrophoretic light scattering (also known as laser doppler micro-electrophoresis) technique in the Zetasizer Nano ZS (Malvern Instruments, Westborough, MA). Samples were suitably diluted in HPLC grade water or 1x PBS, filled into the disposable capillary cell (DTS1070), and analyzed in triplicate.

The phospholipid (DPPC) that were coated onto ANPs at various $W_{\text{Lipid}}/W_{\text{ANPs}}$ % were quantified by colorimetric assay previously published (279). Briefly, a 2 mL portion of 0.1 N ammonium ferrithiocynate solution was added to 1 mL of DPPC in chloroform at various concentrations (0-80 µg/mL). The mixture was vortexed for 2 min, chloroform layer collected, and optical density was read at 464 nm. A calibration curve of DPPC was prepared by plotting optical density versus concentrations of DPPC. To determine DPPC content in CSLAHNPs, an aliquot was added to chloroform, agitated for 2-5 min and optical density of the chloroform layer was measured after addition of 2 mL of ammonium ferrithiocynate solution.

Morphology of the ANPs and CSLAHNPs was characterized by transmission electron microscopy using a negative staining technique. Sequential two droplet method was used for staining samples with 1% w/v sodium silicotungstate on 400-mesh formvar support film on copper specimen grid (Electron Microscopy Sciences, Hartfield, PA) and

dried. The TEM images were acquired using JEOL 2000EX transmission electron microscope (JEOL USA, Inc., Peabody, MA) equipped with high resolution digital camera.

Results and Discussion

In the two-step, ANPs (core) were prepared by desolvation (coacervation) method in the first step (245). In the second step, the lipid film was hydrated with the ANPs dispersion at or above the transition temperature of phospholipids, followed by sonication to obtain CSLAHNPs (87, 88, 133). In the desolvation method, ANPs were prepared by dropwise addition of a desolvating agent (ethanol or acetone) into aqueous albumin solution (adjusted to a particular pH) until the solution became turbid. The turbidity was caused addition of desolvating agent which leads to conformational change in protein structure by reducing the aqueous solubility of albumin which was phase separated to form NPs. At this point, NPs were hardened by addition of gluteraldehyde which crosslinked between ϵ -amino groups of lysine and arginine residues (280, 281). The hydrophilic surface of the ANPs favors formation of phospholipid bilayer with the hydrophilic polar head groups facing towards the hydrophilic albumin core. The process was governed by the ratio of lipids and ANPs (also expressed as $W_{\text{Lipid}}/W_{\text{ANPs}}$). In addition, the input of external thermal and vibrational energy by heat, sonication and agitation increased the mobility and rearrangement of lipids onto the ANPs to form CSLAHNPs.

A number of formulation and process variables were studied during the preparation ANPs by the desolvation method. As mentioned in the introduction, the desired physicochemical properties of blank CSLAHNPs include hydrodynamic mean size around 100-250 nm, PDI < 0.2, and zeta potential < -30 mV. The pH of the albumin solution prior to ethanol addition significantly influenced mean size of the final ANPs (**Figure 2-2**). The mean size drastically decreased from greater than 1 μm at pH 6 to 485 nm at pH 7. At pH 8, the mean size further reduced to around 183 nm. However, further increase in pH to 9 increased size to about 294 nm. The isoelectric point (pI) of the BSA was reported to be 4.7 to 5.4 (242, 282). When pH was closer to the pI of BSA, ANPs became unstable as indicated by mean size of greater than 1 μm . When pH was increased further, carboxyl groups on BSA were ionized to a higher extent. The higher charge density in the ANPs increased the electrostatic repulsion and decreased the particle size at elevated pH (245). The high electronegative charge density was evident from the increased negative zeta potential of ANPs from -15 mV at pH 6 to around -50 mV at pH 9. Similar findings were reported for the optimization of ANPs by the desolvation method (245). Next, the effect of initial albumin concentration on the mean size and polydispersity index (PDI) was studied at pH 8 (**Figure 2-3**). No significant difference in mean size was observed for 5 mg/mL and 20 mg/mL albumin concentrations while size increased at 40mg/mL albumin concentration. At a high albumin concentration, ANPs were close to one another which enhanced coalescence and increase in size. Another possible explanation is the increased viscosity of the solution at higher albumin concentration, which caused increased droplet size after ethanol addition and ultimately

Figure 2-2. Influence of the pH on the mean size and zeta potential of ANPs

The smallest mean size was obtained at pH 8. Values were presented as mean $\pm$ SD (n=3); albumin concentration: 20 mg/mL; ethanol addition rate: 1 mL/min; water/ethanol (v/v): 1:2; glutaraldehyde-to-albumin ratio: 0.47 % w/w; mean size and zeta potential were measured in pure HPLC grade water.

Figure 2-3. Influence of albumin concentration on the mean size and polydispersity index of ANPs

The smallest mean size was obtained at albumin concentration of 20 mg/mL. Values were presented as mean $\pm$ SD (n=3); albumin solution pH: 8; ethanol addition rate: 1 mL/min; water/ethanol (v/v): 1:2; gluteraldehyde-to-albumin ratio: 0.47 % w/w; mean size and PDI were measured in pure HPLC grade water.

leads to increased mean size. of ANPs. Addition of a chemical cross-linker such as glutaraldehyde provides better stability, shape, insolubility at high temperatures, and reduced swelling. In order to study the effect of crosslinking degree on the size and PDI of ANPs, different glutaraldehyde-to-albumin ratios (% w/w) were used during preparation of ANPs (**Figure 2-4**). The glutaraldehyde-to-albumin ratios of 0.188 %, 0.47 %, and 0.94 % w/w corresponded to the 40, 100, and 200 % of the calculated amount necessary for quantitative cross-linking of 59 epsilon amino groups of the lysine in the albumin molecule (230). It was observed that 0.47 % w/w of glutaraldehyde-to-albumin ratio produced smallest mean size of ANPs. The effect of rate of ethanol addition on mean size revealed a higher size at low rate (0.5 mL/min) while reduced size was obtained from higher flow rates of 1 mL/min and 2 mL/min (**Figure 2-5**). Finally the volume ratio of water-to-ethanol showed smallest mean size of ANPs at 1:2 while size increased at 1:3 and 1:4 ratios (**Figure 2-6**).

The thermal stability of the ANPs was performed at 45-50°C since this is above the phase transition temperature of the major lipid component ($T_{m[DPPC]}$ 41.4°C) present in the lipid bilayer (283). Thermal stability data showed no significant change in mean size, PDI, and zeta potential before and after incubation (data not shown). DSC data also confirmed the thermal stability of serum albumin at 45-50°C (284). No significant difference in the physicochemical properties of CSLAHNPs was observed for CSLAHNPs prepared by sonication or extrusion. Sonication was chosen as the preferred method compared to extrusion for the preparation of CSLAHNPs. This is because a significant amount of CSLAHNPs were lost during extrusion method. Unlike liposomes which could deform and pass through the pores of the polycarbonate membrane, the rigid structure of CSLAHNPs possibly clogged the membrane pores and resulted in poor yield. The proportion of total lipids with regard to core ANPs (expressed by lipid-to-ANPs weight ratio or W_{Lipid}/W_{ANPs}) was an important parameter influencing the overall size and stability of CSLAHNPs. At high concentrations of phospholipids, free multilamellar vesicles formed to a greater extent. As a result, the mean size and PDI of CSLAHNPs increased. Furthermore, the purification of final CSLPHNPs was difficult at a very high phospholipid concentration. At low or suboptimal lipid concentrations, there would be insufficient coating on the polymeric core particles, resulting in instability. The number of phospholipid molecules per unilamellar liposome was calculated based on assumptions of spherical geometry of liposomes, 5 nm lipid bilayer thickness, and 0.71 nm² surface area of phospholipid head group (139). Based on the similar assumptions, the W_{DPPC}/W_{ANPs} ratio was calculated to be ~10 % based on the theoretical assumptions of 5 nm DPPC lipid bilayer around spherical 190nm ANPs (core) taking into account the DPPC molecular surface area of the head group (0.628 nm²) (285) and density of BSA (1.32 gm/cc). The effect of W_{Lipid}/W_{ANPs} on the mean size and zeta potential of CSLAHNPs was studied experimentally to validate the calculated value. When W_{Lipid}/W_{ANPs} was increased to 5, 10, 15, 20, 30 %, the mean particle size also increased gradually from 190 nm to around 300 nm (**Figure 2-7**). This can be explained by the formation of multiple lipid layers around the core which increased the mean size of CSLAHNPs. At around 15 % W_{Lipid}/W_{ANPs} (equivalent to ~8 % W_{DPPC}/W_{ANPs}), mean size was around 200 nm which was 10 nm higher compared to the ANPs. Zeta potential value also reduced from -30 mV to about -10mV when W_{Lipid}/W_{ANPs} increased from 0 to 30 %.

Figure 2-4. Influence of glutaraldehyde-to-albumin ratio on the mean size and polydispersity index of ANPs

The smallest mean size was obtained at 0.47 % w/w of glutaraldehyde to albumin. Values were presented as mean $\pm$ SD (n=3); albumin concentration: 20 mg/mL; albumin solution pH: 8; ethanol addition rate: 1 mL/min; water/ethanol (v/v): 1:2; mean size and PDI were measured in pure HPLC grade water.

Figure 2-5. Influence of ethanol addition rate on the mean size and polydispersity index of ANPs

The smallest mean size was obtained at ethanol addition rate of 1 mL/min. Values were presented as mean $\pm$ SD (n=3); albumin concentration: 20 mg/mL; albumin solution pH: 8; water/ethanol (v/v): 1:2; glutaraldehyde-to-albumin ratio: 0.47 % w/w; mean size and PDI were measured in pure grade water.

Figure 2-6. Influence of water-to-ethanol ratio on the mean size and polydispersity index of ANPs

The smallest mean size was obtained at water/ethanol volume ratio 1:2. Values were presented as mean $\pm$ SD (n=3); albumin concentration: 20 mg/mL; albumin solution pH: 8; ethanol addition rate: 1 mL/min; glutaraldehyde-to-albumin ratio: 0.47 % w/w; mean size and PDI were measured in pure grade water.

Figure 2-7. Effect of lipid-to-albumin nanoparticles weight ratio on the mean size and zeta potential of CSLAHNPs

The optimum weight ratio of total lipid to the ANPs was found at 15 % to form the lipid bilayer around core ANPs. Values were presented as mean $\pm$ SD (n=3); mean size and PDI were measured in pure grade water.

The change in zeta potential was indicative of the formation of lipid shell around the ANPs. About 60 mol % of the lipid bilayer was composed on DPPC which is a zwitterionic phospholipid with negative charge on the phosphate group and a positive charge on the amine (286). The net charge of DPPC molecule is close to zero at pH 7.4. Therefore with the increase in lipid concentration, the zeta potential of the CSLAHNPs gradually dropped from -30 mV to -10 mV due to the coating of neutral DPPC around core ANPs. As shown in **Figure 2-7**, the $W_{\text{Lipid}}/W_{\text{ANPs}}$ of ~ 15 % resulted in CSLAHNPs with favorable combination of size (200 ± 4 nm) and zeta potential (-15 ± 2 mV) for drug delivery application. The optimized $W_{\text{Lipid}}/W_{\text{ANPs}}$ of 15 % (which was equivalent to $W_{\text{DPPC}}/W_{\text{ANPs}}$ of ~8 %) obtained experimentally were also in conformity with the calculated $W_{\text{DPPC}}/W_{\text{ANPs}}$ of ~10 %. The concentration of the DPPC adsorbed onto CSLAHNPs was quantified using a colorimetric assay. The data suggested that $W_{\text{DPPC}}/W_{\text{ANPs}}$ of 4.47 ± 1.12 % w/w were adsorbed out of the initial $W_{\text{DPPC}}/W_{\text{ANPs}}$ of 8 %. The difference could be explained by the heterogeneity of ANPs population with various mean sizes which required varied amounts of DPPC to coat the core.

To characterize the structure of CSLAHNPs, they were imaged by transmission electron microscopy (TEM) with negative staining by sodium silicotungstate. Sodium silicotungstate stained the lipid bilayer membrane with high electron density compared to ANPs (core). The TEM image of the ANPs (core) was shown in **Figure 2-8**. The ANPs were smooth and spherical in shape with the absence of dark corona at the edges of particles. **Figure 2-9** showed TEM image of CSLAHNPs with distinct dark corona around the periphery of each CSLAHNP. The thickness of the layer was measured around 5 nm which confirmed the presence of phospholipid bilayer (shell) around the core (287). The CSLAHNPs were smooth and spherical in shape with some degree of polydispersity as seen with core ANPs in **Figure 2-8**. The particle size observed in TEM was in good agreement with that obtained by DLS. A slight reduction in size was observed in TEM compared to DLS. This is because DLS provides the statistical Z-average hydrodynamic size based on the diffusion coefficient of particles in the liquid phase using the Stokes-Einstein equation whereas TEM provides estimation of the projected area diameter of particles in the dried state under high vacuum (288).

Summary and Conclusion

Blank CSLAHNPs with albumin core and phospholipid bilayer shell were successfully designed, prepared and optimized by the two step method using bovine serum albumin, DPPC, cholesterol, and DSPE-PEG2000 as the formulation ingredients. All the ingredients used were biodegradable, biocompatible, non-immunogenic or mildly immunogenic and approved by FDA for human use. The formulation and process variables were identified and optimized in the preparation of CSLAHNPs by the two-step method. Various factors influenced the physicochemical properties including mean size, polydispersity index, and zeta potential of both ANPs (core) and CSLAHNPs. In the two-step method, a number of variables were identified and optimized for the preparation of ANPs (core) by the desolvation method (first step); albumin concentration 20 mg/mL, pH of the albumin solution at 8, ethanol addition rate 1 mL/min, volume ratio

Figure 2-8. Transmission electron micrograph of the ANPs (core)

ANPs were spherical and uniform with a low population of smaller particles.

Figure 2-9. Transmission electron micrograph of CSLAHNPs

The sample was stained with 1 % sodium silicotungstate. The particles showed smooth and spherical shapes with moderate degree of polydispersity. The lipid shell appeared as a dark corona around each particle.

of water to ethanol 1:2, and glutaraldehyde-to-albumin ratio 0.47 %w/w. In the second step, DPPC, cholesterol, and DSPE-PEG2000 were used at a molar ratio of 60:30:10 for the preparation of CSLAHNPs by the lipid film hydration method. The critical parameter was identified as the weight ratio of lipid-to-ANPs ($W_{\text{Lipid}}/W_{\text{ANPs}}$) during the optimization of CSLAHNPs. The experimental $W_{\text{Lipid}}/W_{\text{ANPs}}$ was found around 15 % w/w (equivalent $W_{\text{DPPC}}/W_{\text{ANPs}} \sim 8$ % w/w) which was close to the calculated value (~ 10 % w/w) based on the theoretical assumptions. TEM images showed smooth and spherical particles of both ANPs (core) and CSLAHNPs with some degree of polydispersity. A distinct electron dense corona around periphery of the CSLAHNPs was visible which was absent in ANPs.

CHAPTER 3. PREPARATION, CHARACTERIZATION, AND OPTIMIZATION OF ERLOTINIB LOADED CORE SHELL TYPE LIPID ALBUMIN HYBRID NANOPARTICLES

Introduction

Lung cancer, particularly non-small cell lung cancer (NSCLC) is the leading cause of cancer related mortality in both men and women worldwide (2). Erlotinib hydrochloride (ETB), an EGFR tyrosine-kinase inhibitor, promotes cell cycle arrest and apoptosis, and leads to the inhibition of angiogenesis and cell invasion (16). ETB was approved as the second or third line therapy in patients with locally advanced or metastatic NSCLC after failure of at least one chemotherapy regimen (17). ETB is available on the market as oral film-coated tablet 25-150 mg, sold under the trade name Tarceva[®], and showed oral bioavailability of 60 % (289). NSCLC treatment requires long-term administration with a minimum of 4-6 cycles and the drug is likely to attack both cancerous and normal cells. As a consequence of long term therapy, toxic effects were observed including severe skin rash and diarrhea. No injectable formulation of ETB is available in the market, although such a form would be useful for patients with gastrointestinal abnormalities (27).

The development of an injectable formulation of ETB would be useful for administering the drug in patients with NSCLC. Nanoencapsulation of anticancer drugs in colloidal nanocarriers offers the advantages of more favorable pharmacokinetics, drug protection, the enhancement of therapeutic efficacy, the reduction of potential toxic side effects and the increase of patient comfort by avoiding repetitive bolus injections (40, 290-292). A recent study showed a reduction in sub-acute toxicity in mice after intravenous administration of ETB loaded in PLGA NPs (223). Among various nanoparticulate drug delivery systems, CSLAHNPs have emerged as a promising drug delivery platform effective for anticancer agents, antibiotics, proteins, and genes. The system showed advantages of encapsulation of multiple drugs, increased overall drug payload, controlled drug release profiles, and improved stability (65). Many small molecular weight chemotherapeutic drugs (135, 138), antibiotics (141), proteins (91) and nucleotides (103, 194) have been encapsulated or entrapped in CSLPHNPs (70, 277, 293-300). CSLPHNPs can be loaded with either a single drug or two drugs (133, 145, 301). For single drug loaded CSLPHNPs, the drug can be loaded into both the polymeric core as well as in the lipid shell, thereby increasing the total drug payload in the system. For two drugs, CSLAHNPs can also be co-loaded with one drug in the core and the other one in the shell (145). This is a unique feature of CSLPHNPs which can combine multiple functionalities in a single delivery platform. There are two commonly used strategies for drug loading in CSLPHNPs; incorporation method and adsorption method. In the incorporation method, drug(s) is/are incorporated during the preparation step i.e. in the core and/or in the lipid film. Another option is to adsorb or absorb the drug with the cores and lipid vesicles separately before combining to form CSLPHNPs. However, the DL is generally expected to be better in the incorporation approach than the adsorption approach (146). The adsorption method has been used to load DNA into lipoparticles

composed of PLA core/DPPC-DPTAP lipid shell (103). The macromolecules or proteins show greatest loading efficiency near its isoelectric point when it has minimum solubility and maximum adsorption (147). For small molecules, the use of ionic interactions between the drug and polymer can be an effective way to increase the drug loading (96). Examples of the factors which may influence the DL and EE are aqueous solubility of the drug, affinity and miscibility of the drug in both polymer and lipid phases (96), amount of lipid (144), drug-lipid charge interactions (141), aqueous phase pH (148), and methods of preparation. Often it is required to carry out in-depth physicochemical characterization during preformulation studies to optimize LC and EE (149). For instance, Li *et al* have analyzed the combined solubility parameters and partition coefficients for screening the best lipid and polymer for the highest LC and the maximum binding capacity to the cationic drug verapamil (149). Based on the approach, they have reported drug EE greater than 90 % and DL between 5 to 36.1 % (96). The amount of the lipid is also a decisive factor for EE of lipophilic drugs in CSLPHNPs. Liu *et al* reported the decrease in EE from 42 % to 15% when the lipid component was lowered from 0.1 % to 0.01 % for paclitaxel particles (144). Drug lipid charge interactions may be important for encapsulation of drugs. Cheow *et al* reported successful encapsulation of zwitterionic levofloxacin and ofloxacin when PLGA polymer and phosphatidyl choline (PC) lipid were used, whereas formation and loading of cationic ciprofloxacin into the CSLPHNPs was unsuccessful (141). When PC was replaced with non-ionic polyvinyl alcohol, ciprofloxacin loaded CSLPHNPs were successfully produced. The results suggested the possibility of unfavorable ionic interactions between the anionic PC and cationic ciprofloxacin for the failed formulation. The method of preparation also affects the DL and EE. The method used during core PNPs preparation, such as solvent displacement method leads to poor DL and EE for hydrophilic compounds (150). Another problem of the two-step method is that encapsulated drugs leak out before the lipid coat is formed (141).

For the present work, bovine serum albumin (BSA) was selected as the core material with phospholipids (DPPC, cholesterol, DSPE-PEG2000) as the shell for the loading of ETB in CSLAHNPs. Serum proteins including albumin offer several advantages; biodegradability, biocompatibility, non-antigenic, easy to prepare, controlled particle size distribution, various possibilities for surface modification, greater stability, relatively easy to scale up and binding ability for a number of hydrophilic and lipophilic drug molecules (226, 227). BSA is a well-known globular protein which has been well-investigated over the past 40 years (302). The molecule consists of three intrinsic fluorophores; tryptophan (trp), tyrosine, and phenylalanine with two trp residues; trp-212 and trp-134 (243). Trp-212 is located within a hydrophobic binding pocket and Trp-134 on the surface of a molecule(243). The interaction and binding of drugs with albumin is of great importance in various biomedical applications, particularly drug delivery and receptor targeting. ETB-loaded CSLAHNPs (ETB-CSLAHNPs) with high drug payload would be advantageous in maximizing the therapeutic efficacy of ETB for the therapy of NSCLC. The objective of the study was to optimize drug loading strategies in CSLAHNPs along with investigation of the formulation and process variables that influence physicochemical properties of ETB CSLAHNPs.

Experimental Section

Materials

Bovine serum albumin (BSA, Cohn fraction V, lyophilized powder, MW~66000, purity 95-99 %) and glutaraldehyde solution (25 % in water) were obtained from Sigma-Aldrich (St. Louis, MO). Erlotinib hydrochloride (purity 99 %) was obtained from LC Laboratories (Woburn, MA). The lipids used in this work were all of research grade. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N (poly(ethylene glycol)2000) [DSPE-PEG2000] were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol, ferric chloride hexahydrate, and ammonium thiocyanate were purchased from Sigma (St. Louis, MO). High performance liquid chromatography (HPLC) grade water, ethanol, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). HPLC grade chloroform and 1N sodium hydroxide solution were purchased from Acros Organics (Morris Plains, NJ). All other chemicals and reagents were of analytical grade and used without further purification or characterization.

Quantification of erlotinib

A sensitive reversed phase HPLC method was developed and validated to quantitate ETB in the CSLAHNPs. HPLC system consisted of a Waters 600 controller, Waters 717 plus auto sampler and a Waters 2996 photodiode array detector. Data were acquired and processed with Waters Millennium 32 software (version 4.0). Chromatographic separation was achieved on a NovaPak® C18 reverse phase column (3.9 X 150 mm) from Waters (Milford, MA). The isocratic mobile phase consisting of acetonitrile and acidified water pH 2.6 (40:60, v/v) was pumped at a flow rate of 1.0 mL/min with an injection volume of 20 µL. ETB (retention time, 5.0 min) was monitored at 346 nm with a photodiode array detector.

Optimization of drug binding to albumin

The drug binding capacity of albumin was determined experimentally. The various parameters or variables that affect drug binding with BSA were investigated and optimized to obtain ETB-ANPs with highest drug loading capacity. Briefly, albumin solution (1 mg/mL) and aliquots of ETB (drug) in DMSO were added and incubated in various pH conditions for 0-24 h at both room temperature (RT) and at 37°C. At predefined time intervals, the sample was filtered through Amicon® Ultrafiltration device (MWCO 30 kDa) after addition of twice the volume of acetonitrile/water (40:60) mixture to extract the unbound drug in the filtrate. Drug concentration in the filtrate was determined by HPLC method stated above.

Preparation of ETB-ANPs

Erlotinib loaded ANPs (ETB-ANPs) were prepared by two different methods: incorporation method and absorption method. The method that provided highest drug loading efficiency was chosen.

In the incorporation method, albumin was dissolved in water and drug dissolved in DMSO as cosolvent. Then, albumin solution and drug solution were incubated together at various ratios of drug to albumin: 2, 4, 8, 12 or 24 h at different pH conditions (3.4, 5.4 or 7.4) and at different temperature conditions (20 °C or 37°C). Drug loaded ANPs were prepared by first adjusting the pH of the albumin drug mixture to pH 8-9, followed by addition of ethanol dropwise at 1 mL/min until turbidity appeared. The nanosized droplets or coacervates formed were hardened for 12 h after adding glutaraldehyde solution (0.47 % w/w of BSA) and the resulting ETB-ANPs were purified by centrifugation at 12000 rpm for 30 min (159). ETB-ANPs were also washed thrice.

Drug loading in ANPs can be accomplished by ad/absorption method because of the presence of drug binding sites in albumin molecules (303, 304). In the ad/absorption method, ANPs (core) was first prepared by desolvation method as discussed in the previous chapter. Then, ANPs and drug solution in DMSO were incubated together at various drug to albumin ratios, 12 or 24 h, at different pH conditions (3.4, 5.4 or 7.4) and at different temperature conditions (20 or 37°C). The ETB-ANPs were purified by centrifugation at 12000 rpm for 30 min (159).

Preparation of ETB-CSLAHNPs

ETB-ANPs that were prepared in the previous step were used in the preparation of ETB-CSLAHNPs. About 5 % w/w of additional drug/lipids was incorporated into the lipid film. Drug and lipids (DPPC, cholesterol, DSPE-PEG2000; molar ratio 60:30:10) were dissolved in a small quantity of methanol/ethanol (50:50 v/v) in a round bottom flask and evaporated in vacuum evaporator for 1-2 h to form dry lipid film. The lipid film was hydrated with ANPs dispersion in 1x PBS) for 15-30 min at 45-50°C. The dispersion was sonicated in a bath sonication for 10 min. The optimized lipids to ETB-ANPs ratio of 15 % w/w was used as described in Chapter 2.

Purification of ETB-CSLAHNPs

The free vesicles and micelles were removed by ultracentrifugation cycle at 10000-14000 rpm for 20-30 min, followed by washing thrice. The unbound free drug and other small molecular weight species were removed by gel filtration column (Sephacrose CL 4B) by spinning the sample at 400 g for 5 min with 1x PBS as the elution buffer. The final product was reconstituted in 1x PBS.

Characterization of ETB CSLAHNPs

The mean diameter, PDI, and zeta potential of the ETB-CSLAHNPs were determined using the methods reported in Chapter 2. Particle surface morphology and shape of the ETB-CSLAHNPs were characterized by TEM as mentioned in Chapter 2.

In order to examine the particle surface morphology and shape, scanning electron microscopy (SEM) was also used. A concentrated aqueous suspension was spread over a slab and dried under vacuum. The sample was shadowed in a cathodic evaporator (also known as “sputter coater”) with a gold layer of 20 nm thick in an argon gas environment at 45 mA current for 5 seconds. Photographs were taken using a JSM-5200 Scanning Electron Microscope (Tokyo, Japan) operated at 10 kV.

Yield of CSLAHNPs was determined by microgravimetry analysis (305). An aliquot (50-100 μ L) of the CSLAHNPs dispersion was put in an aluminium pan, dried for 2h at 80°C, and cooled off. Subsequently, the pans were weighed with a microbalance. The yield (mg/mL) was calculated from the difference in weight of the empty and CSLAHNPs filled pan.

Drug entrapment efficiency (DEE) and drug loading (DL) of ETB-CSLAHNPs were quantified by HPLC method as reported earlier in this chapter. For comparison, DEE and DL of ETB-ANPs (core) were also quantified by HPLC method. The drug was extracted from CSLAHNPs/ANPs using acetonitrile/water (40:60 v/v) as solvent under sonication for 30-45 min. After centrifugation, the supernatant was injected in HPLC system to determine the quantity of ETB using the method previously reported. DEE and DL were calculated by the **Equation 3-1** and **Equation 3-2** respectively.

$$DEE (\%) = \frac{\text{Mass of drug in nanoparticles}}{\text{Total mass of drug used}} \quad (\text{Eq. 3-1})$$

$$DL (\%) = \frac{\text{Mass of drug in nanoparticles}}{\text{Total mass of drug loaded nanoparticles}} \quad (\text{Eq. 3-2})$$

Transmission infrared spectra were recorded on a Fourier transform infrared (FTIR) spectrophotometer (Perkin-Elmer Spectrum BX, Perkin-Elmer Inc., Norwalk, CT, USA). The FT-IR spectra were obtained by averaging 32 scans at a resolution of 2 cm^{-1} .

Thermal analysis was conducted using DSC 2010 differential scanning calorimeter (TA Instruments, New Castle, DE). The temperature and heat flow calibrations were performed at a heating rate of 5°C/min from 0 to 200°C with indium as a standard substance. Powder samples, 1–5 mg each, were analyzed at the same settings under a purge of nitrogen (50 mL/min). Each analysis was performed in triplicate.

Drug release was quantified in 4000 mL of dissolution media (PBS pH 7.4 and acetate buffer pH 5.4) using dialysis method previously reported (69). The in-house set up for *in vitro* drug release experiment was shown in **Figure 3-1**. A 100 μ L portion of the

Figure 3-1. Schematic diagram of the in-house experimental setup for *in vitro* drug release study

The ETB-CSLAHNPs liquid dispersion was equally divided and placed in minidialysis tubes (MWCO 3.5 k Da). The tubes were put inside the floater and placed in a beaker containing dissolution medium kept on a magnetic stirrer to agitate the medium. The beaker and the magnetic stirrer were kept inside an environmental chamber maintained at 37°C.

ETB-CSLAHNPs dispersion in 1x PBS were pipetted out and added into each Slide-A-Lyzer MINI dialysis microtubes with a molecular weight cutoff of 3500 Da (Pierce, Rockford, IL). Minidialysis tubes were kept in a floater which was placed in a beaker containing 4000 mL of the dissolution medium (PBS pH 7.4 or acetate buffer pH 5.4) under magnetic stirring at 75 rpm at 37°C. The entire set up was kept inside a temperature controlled oven. At predefined time point, dialysis tubes containing samples were taken out, extracted in acetonitrile/water (40:60 v/v) with sonication or vortexing for 0.5-1 h at room temperature. After centrifugation, the samples were injected in HPLC to quantify the amount of drug released in specified time points. Data of the ETB release from ETB-CSLAHNPs were analyzed for release kinetics by zero-order, first order, Higuchi, Hixon-Crowell, and Korsmeyer-Peppas model (306, 307).

The colloidal stability of CSLPHNPs in serum was performed according to the previously published absorbance (93, 125). CSLAHNPs were suspended in 50 % fetal bovine serum (FBS) with a final nanoparticle concentration of 1 mg/mL (dispersed in 1x PBS). In order to do this, particles were washed in PBS using Amicon® Ultrafilter and concentrated to 2 mg/mL. An equal volume of FBS was then added. Samples were incubated at 37°C with periodic light shaking. At each time point, an aliquot of NP solutions was at predetermined time points (0, 0.5, 1, 2, 3, 4, and 6 h). Due to interference of high concentration of plasma proteins at 50 % FBS, absorbance measurements were conducted at 560 nm for each time point using a UV-Visible Spectrophotometer. The measurements were performed in triplicate at room temperature.

Results and Discussion

For the purpose of rational design of ETB-CSLAHNPs, preformulation characterization studies were performed. ETB is a quinazolinamine derivative with the molecular weight of 429.9, pKa of 5.42 at 25°C with pH dependent solubility profile (9). Increased aqueous solubility was observed at a pH of less than 5 due to protonation of the secondary amine (9). The pH of maximum solubility occurs at pH 2. Pharmacokinetics data showed 95 % binding with plasma proteins in humans (9). Binding of ETB with bovine serum albumin (BSA) was studied (308, 309). BSA has three homologues domains (I, II, III) with each domain having two sub-domains (A and B) and 17 disulfide bonds (310). Human serum albumin (HSA) also has three domains (I, II, III) with each having two subdomains and stabilized by 17 disulfide bridges (243). The major drug binding sites in BSA and HSA are located in the hydrophobic cavities in subdomains IIA and IIIA (311, 312). Because of the structural similarities with HSA, BSA has been frequently used as a model protein for drug binding studies (313). The binding of ETB with BSA was investigated by Liu et al (308). Using NMR, spectroscopic, thermodynamic and molecular modeling methods, they reported that ETB was bound to domain II of BSA with high affinity through hydrogen bond and van der Waals forces. Rasoulzadeh *et al* also reported the formation of ETB-BSA complex through hydrophobic interaction and the presence of single ETB binding site on BSA (309). Therefore, BSA was selected as the core forming material of the CSLAHNPs for the loading and delivery of ETB.

The ETB binding study was performed to select and optimize the formulation and process variables for the subsequent preparation of ETB-ANPs. The unbound ETB was quantified by HPLC method using the standard curve which was linear between 0.625 and 80 $\mu\text{g/mL}$ with a coefficient of determination (r^2) value of 0.9999. The effect of pH of the incubation medium was studied at room temperature (RT~20°C) and at 37°C for 4h (**Figure 3-2**). The results exhibited highest ETB binding with BSA at pH 7.4 and pH 5.4 while binding capacity decreased at acidic pH 3.4. No significant difference was observed for RT and 37°C incubation. This observation could be explained by pH-ionization hypothesis. The isoelectric point (pI) of BSA is ~ 4.7-5.4 at 25°C. ETB has a pKa of ~ 5.4. BSA molecules exist as predominantly cationic below its pI. ETB is a weakly basic compound and exists as highly ionized (cationic) with protonation of secondary amine. Therefore, electrostatic repulsion of ETB and BSA molecules reduced their interaction at pH 3.4. At pH 5.4, 50 % of ETB would ionize while remaining 50 % would be unionized. Therefore, the hydrophobic interaction and ionic interaction between ETB and BSA lead to increased drug binding capacity. At pH 7.4, ETB would be maximally unionized and the hydrophobic interaction between drug and BSA was predominant. The influence of incubation time on drug binding to albumin was studied at RT and 37°C while keeping the pH of the incubation medium at 7.4 (**Figure 3-3**). Higher drug binding was observed at RT compared to 37°C for 1 h to 24 h. Maximum drug was bound to albumin at 8 h, pH 7.4 and at RT condition. The effect of drug-to-albumin molar ratio on the drug binding capacity was shown in **Figure 3-4**. Maximum drug binding occurred at drug-to-albumin molar ratio of 10:1 after 8h at pH 7.4.

ETB was encapsulated in the CSLAHNPs to develop a prototype formulation for drug delivery to NSCLC. The preparation, characterization and optimization of CSLAHNPs (without ETB) were described in detail in Chapter 2. ETB-CSLAHNPs were also prepared by the two-step method with two different strategies for loading the drug (ETB) into CSLAHNPs; incorporation and sorption method. In the incorporation method, ETB was added to BSA solution prior to addition of ethanol to form ETB-loaded ANPs. In the sorption method, ANPs were first prepared by desolvation method, followed by incubation with ETB to form ETB-ANPs. **Figure 3-5** showed the drug loading (DL %) and mean size of ANPs as a function of incubation time using incorporation method. However, DL was poor with no significant difference observed between two incubation times (4 h, 8 h, and 12 h) and two temperatures (RT and 37°C). The addition of high volume of ethanol might have solubilized ETB during particle formation and significant amount of drug was lost in the subsequent purification of ANPs. Sorption method showed higher DL in ANPs at 12 h and RT incubation (**Figure 3-6**). This is because the drug was added after the preparation of ANPs and the desolvating agent (ethanol) did not interfere with DL of ANPs. In the method, a part of the drug was absorbed while other parts were adsorbed on the surface of ANPs, increasing the final DL. Therefore, sorption method was selected for the preparation of ETB-ANPs.

After the preparation of ETB-ANPs, additional ETB (5 % w/w ETB/lipid) was also incorporated in the lipid bilayer (composed of DPPC, cholesterol, DSPE-PEG2000 molar ratio 60:30:10) to increase DL and DEE of the final ETB-CSLAHNPs. The physicochemical properties of the optimized ETB-ANPs and ETB-CSLAHNPs were

Figure 3-2. The influence of pH on the drug binding to albumin in solution at room temperature (RT~20°C) and at 37°C for 4h

The data were presented as mean $\pm$ SD (n =3). The highest ETB binding with BSA at pH 7.4 and pH 5.4 while binding capacity decreased at acidic pH 3.4. No significant difference was observed for RT and 37°C incubation.

Figure 3-3. The effect of incubation time on drug binding to albumin in solution

The pH of the solution was kept at 7.4. The study was performed at two temperature conditions: RT (20°C) and 37°C. The data were presented as mean $\pm$ SD (n=3). Higher drug binding was observed at RT compared to 37°C for 1 h to 24 h. Maximum drug was bound to albumin after 8 h, pH 7.4 and at RT condition.

Figure 3-4. The influence of drug-to-albumin molar ratio on drug binding to albumin in solution after 8 h. at pH 7.4

The data were presented as mean $\pm$ SD (n =3). Maximum drug binding occurred at drug-to-albumin molar ratio of 10:1 after 8 h at pH 7.4.

Figure 3-5. Influence of drug loading and mean size of ETB-ANPs prepared by incorporation method as a function of incubation time at RT and 37°C

The data were presented as mean $\pm$ SD (n =3). In the incorporation method, DL was poor with no significant difference observed among 4, 8, and 12 h at two temperatures (RT and 37°C).

Figure 3-6. Influence of drug loading and mean size of ETB-ANPs prepared by sorption method as a function of incubation time at RT and 37°C

The data were presented as mean $\pm$ SD (n =3). Sorption method showed higher DL in ANPs at 12 h and RT incubation.

shown in **Table 3-1**. ETB-CSLAHNPs were 194 nm in size with PDI 0.16, zeta potential -15.6 mV, yield 83.5 % w/w, DL 2.08 % w/w, and DEE 32.75 % w/w.

TEM photomicrograph of ETB-CSLAHNPs was shown in **Figure 3-7**. Sodium silicotungstate was used for negative staining. ETB-CSLAHNPs were smooth and spherical in shape with distinct dark lipid corona around core. Similar observation was reported for TEM image of CSLAHNPs in chapter 2. Additionally, SEM image was taken for these particles which also showed spherical particles with low to moderate degree of polydispersity (**Figure 3-8**). As mentioned in Chapter 2, a reduction in size was observed in both TEM and SEM images compared to DLS. This is because DLS provides the statistical Z-average hydrodynamic mean size based on the diffusion coefficient of particles in the liquid phase using the Stokes-Einstein equation whereas TEM and SEM provide estimation of the projected area diameter of particles in the dried state under high vacuum (288).

FT-IR analysis was used to characterize any chemical interaction that occurred in the hybrid NPs system among the drug, albumin and lipids. The FT-IR spectra of ETB, BSA, DPPC, cholesterol, DSPE-PEG2000, physical mixture of ETB and BSA, ETB-ANPs and ETB-CSLAHNPs were shown in **Figure 3-9**. ETB peaks found at 649, 867, 901, 948, 1029, 1073, 1213, 1242, 1355, 1431, 1484, 1624, and 3401 cm^{-1} . The FT-IR spectra of native ETB illustrated characteristic bands due to different functional groups such as 3495, 1637, 1509 and 1024 cm^{-1} , corresponding to OH stretching vibration, NH stretching, aromatic C-C stretching and C=O stretching, respectively. The spectra of ETB-CSLAHNPs showed the presence of bands similar to the physical mixture of ETB and BSA. Therefore, it can be inferred that ETB-CSLAHNPs system showed no significant adverse chemical interaction among the constituents.

DSC studies were carried out to determine whether the drug was incorporated in the CSLAHNPs as crystalline, amorphous, or bound form. In **Figure 3-10**, the DSC thermograms demonstrated that pure ETB had a sharp endothermic melting peak at 235°C. Pure BSA powder showed two endothermic peaks: smaller peak at ~150°C and bigger peak around 200°C. DPPC melted around 65°C and pure DSPE-PEG2000 showed dual endothermic peaks (40°C and 50°C). ETB-CSLAHNPs showed endothermic peaks corresponding to BSA and lipids with no peak for ETB. This suggested that the encapsulated ETB might exist in the BSA and lipid matrix as either amorphous form or in disordered crystalline phase (314).

Figure 3-11 showed the plot of the drug release data expressed as percentage drug released from ETB-CSLAHNPs in two dissolution media (PBS pH 7.4 and acetate buffer pH 5.2) as a function of time. The encapsulated ETB was released from the CSLAHNPs at a sustained rate over 96 h. When PBS pH 7.4 was used as the dissolution medium, ETB-CSLAHNPs showed a biphasic release pattern with initial fast release followed by slower release up to 96 h. The initial faster release of around 60 % of the encapsulated drug was observed in 24 h, followed by slower release of drug from CSLPHNPs up to about 100 % in 96 h. The initial faster drug release could be attributed

Table 3-1. Physicochemical properties of the optimized ETB-ANPs and ETB-CSLAHNPs

Batch	Mean Size (nm)	PDI	Zeta potential (mV)	Yield (% w/w)	DL (% w/w)	DEE (% w/w)
ETB-ANPs	185 ±8	0.13±0.01	-25.1±3.9	71.3±3.5	1.42±0.5	24.95±7.73
ETB-CSLAHNPs	194 ±6	0.16±0.02	-15.6±4.1	83.5±5.9	2.08±0.7	32.75±5.34

Data presented as mean ±standard deviation (n=3). In ETB-CSLAHNPs, lipids were composed of DPPC, cholesterol, DSPE-PEG2000 (molar ratio 60:30:10). In ETB-ANPs, initial drug-to-albumin molar ratio was kept at 10:1 (6.45% w/w drug/albumin).

Figure 3-7. TEM image of ETB-CSLAHNPs

Scale bar represents 100 nm. TEM image revealed spherical and smooth shape of ETB (drug) loaded CSLAHNPs with dark corona of lipid bilayer. The image was similar to TEM image of the blank CSLAHNPs as shown in Chapter 2.

Figure 3-8. SEM image of ETB-CSLAHNPs

Scale bar represents 200 nm. SEM image revealed spherical and smooth shape of ETB (drug) loaded CSLAHNPs.

Figure 3-9. FTIR spectra of CSLAHNPs and its components

(A) physical mixture of ETB and BSA, (B) ETB, (C) ETB-ANPs, (D) ETB.HCl, (E) ETB-ANPs, (F) ETB-CSLAHNPs, (G) BSA, (H) Cholesterol, (I) DPPC, and (J) DSPE-PEG2000. FTIR revealed no adverse chemical interaction was present in the CSLAHNPs containing drug.

Figure 3-10. DSC thermograms of CSLAHNPs and its components

(A) ETB. HCl, (B) ETB-CSLAHNPs, (C) BSA, (D) DPPC, (E) DSPE-PEG2000, and (F) Cholesterol. DSC results indicated the existence of drug in the CSLAHNPs as amorphous or molecularly dispersed solid solution state.

Figure 3-11. *In vitro* drug release profiles of ETB-CSLAHNPs in pH 7.4 and acetate buffer pH 5.2

The data were presented as mean $\pm$ SD (n=3). Sustained release of drug from CSLAHNPs was observed for about 72-96 h. Drug release rate was faster in pH 5.2 compared to pH 7.4.

to the presence of drug at or near the surface of CSLPHNPs. The burst release effect was absent probably due to the presence of hydrophobic lipid bilayer around the ANPs which acted as the diffusion controlled membrane. The biphasic drug release pattern was reported for ANPs and surfactant coated ANPs in the literature (234, 315). BSA was used as the core forming biodegradable polymer which was reported to be highly permeable for low molecular weight drugs. The predominant mechanism of drug release from BSA matrix occurred through diffusion rather than polymer degradation. However, the burst release effect was absent due to the presence of hydrophobic lipid bilayer which acted as the diffusion controlled membrane. When acetate buffer pH 5.2 was used as the dissolution medium, the drug release rate was faster compared to PBS pH 7.4, indicating pH sensitive release of ETB-CSLAHNPs (**Figure 3-11**). After 24 h, ETB-CSLAHNPs released about 80 % drug in pH 5.2 while about 60 % drug release was observed at pH 7.4. After 48 h, drug release was around 100 % at pH 5.2 while only about 66 % drug was release at pH 7.4. Significant difference between drug release profiles at two different pH media was observed from after 6 h up to 96 h. The pH sensitivity of ETB-CSLAHNPs might be beneficial for enhanced drug release in the acidic microenvironment of tumors and also the acidic endosome/lysosome compartments of cells (316-318). **Table 3-2** showed the coefficient of determination and release rate constants calculated from in vitro drug release data using various drug release kinetic models (zero order, first order, Higuchi, Hixon-Crowell, and Korsmeyer-Peppas). From the drug release profiles at pH 7.4, Higuchi and 1st order kinetic models fitted best ($r^2 \sim 0.97$). This indicated that the drug release from CSLAHNPs occurred through diffusion controlled mechanism. Drug release profile at pH 5.2 fitted best with 1st order kinetics ($r^2 \sim 0.92$). Therefore, it can be interpreted from first order release kinetics that the drug release rate was dependent on the concentration of drug in CSLAHNPs. Similar findings were reported for biphasic release of 5-fluorouracil loaded ANPs (319). Moreover, the Korsmeyer-Peppas release model showed high correlation for drug release from ETB-CSLAHNPs at both pH values. The diffusion exponent, n , was about 0.8, which confirmed that the anomalous diffusion or non-fickian diffusion was the controlling factor in drug release. Therefore, drug release from CSLAHNPs was governed by both diffusion and erosion of matrix as indicated by non-fickian diffusion mechanism (320).

Stability of CSLAHNPs in serum is an important consideration for their usefulness of CSLAHNPs as the drug delivery system *in vivo* where dilution after intravenous administration could result in NPs aggregation, disassembly and/or drug leakage. CSLAHNPs were incubated in 50 % serum at 37°C with gentle stirring followed by determination of the absorbance at 560 nm. Absorption method was used since CSLAHNPs cannot be accurately detected in dense serum solution by dynamic light scattering technique due to the matrix protein interference from serum (321). Aggregation phenomena were characterized by increased absorbance to higher values. The interaction of CSLPHNPs with various serum proteins in the incubation media would cause neutralization of zeta potential of NPs. The process subsequently would reduce repulsion energy between particles and progressively facilitate agglomeration process and the formation of larger particles. As shown in **Figure 3-12**, ETB-CSLAHNPs were stable in serum retaining the integrity up to 6 h as indicated by no significant change in absorbance values at 560 nm similar to ETB-ANPs.

Table 3-2. *In vitro* release kinetics of ETB-CSLAHNPs

Release model	r^2/K	pH 7.4	pH 5.2
Zero Order	r^2	0.8874	0.7156
	K_0	0.9127	0.9789
First Order	r^2	0.9795	0.9214
	K_1	-0.0099	-0.0183
Higuchi	r^2	0.97	0.8816
	K_H	9.9221	11.298
Hixon-Crowell	r^2	0.9619	0.857
	K_{HC}	-0.0252	-0.0361
Korsmeyer-Peppas	r^2	0.9626	0.9652
	n	0.7841	0.8073

r^2 = Coefficient of determination. K_0 = Zero-order release rate constant. K_1 = First-order release rate constant. K_H = Higuchi release rate constant. K_{HC} = Hixon-Crowell release rate constant. n = diffusion exponent.

Figure 3-12. *In vitro* stability of ETB-CSLAHNPs and ETB-ANPs in 50% fetal bovine serum

The data were presented as mean $\pm$ SD (n=3). Particles retained their physicochemical properties without aggregation for a prolonged period of time (6 h).

Summary and Conclusion

The drug binding capacity of albumin in solution was studied and optimized at various pH conditions, durations of incubation, incubation temperatures, and drug-to-albumin molar ratios. The maximum drug binding capacity of albumin was observed at 6 h incubation in pH 7.4 at room temperature with drug-to-albumin molar ratio 10:1. ETB-CSLAHNPs were successfully designed, prepared and optimized by the two step method as described in Chapter 2. Two strategies for drug loading in ANPs were studied; incorporation and sorption method. Sorption method provided better drug loading and entrapment efficiency in ETB-ANPs compared to incorporation method. Additional ETB was incorporated in the lipid bilayer to maximize the drug loading and entrapment efficiency of ETB-CSLAHNPs. ETB-CSLAHNPs were ~194 nm in size with PDI ~0.16, zeta potential -15.6 mV, yield 83.5 % w/w, DL 2.08 % w/w, and DEE 32.75 % w/w. SEM and TEM images showed smooth and spherical particles of ETB-CSLAHNPs. Drug release from ETB-CSLAHNPs occurred in a sustained manner up to 96 h with a biphasic release pattern in pH 7.4. Drug release was faster in acidic pH of 5.2. Drug release kinetics analysis showed a first order release kinetics with non-fickian diffusion mechanism. ETB-CSLAHNPs were stable in serum for up to 6 h. Therefore, the optimized ETB-CSLAHNPs would be utilized for the conjugation of monoclonal antibodies for the preparation of targeted ETB-CSLAHNPs as described in the following chapter.

CHAPTER 4. DEVELOPMENT OF HALF-ANTIBODY CONJUGATED ERLOTINIB LOADED CORE SHELL TYPE LIPID ALBUMIN HYBRID NANOPARTICLES

Introduction

Conventional chemotherapy of cancer has often exhibited limited therapeutic efficacy along with severe systemic toxicities (322). As an alternative, small molecule EGFR tyrosine kinase inhibitors (TKI) such as ETB that target overexpressed EGFR present on the NSCLC tumor blood vessels endothelial membranes have been used clinically as the molecularly targeted therapy (323). However, EGFR TKI therapy can cause serious toxicities because they are not organ or tissue specific (324). After binding with growth factors such as EGF, EGFR elicits the internalization and intracellular sorting of receptor-ligand complexes and activates signaling pathways (325). The endocytosis of ligand-activated EGFR was found to be EGF concentration dependent. At low concentration of ligand (EGF), it involved clathrin-mediated endocytosis (326). At high EGF concentration, caveolae-mediated endocytosis was prevalent (327). The ligand-EGFR complex undergoes intracellular trafficking and finally degrades in lysosomes after ubiquitin-dependent sorting in early endosomes (325). The internalization and lysosomal trafficking phenomena can be utilized by directing TKI to specific tumor cells *via* passive targeting and/or active targeting strategies. Therefore, the efficacy of TKI could be improved by targeted delivery and release of small molecule EGFR TKI inside the tumor cells using anti-EGFR antibody conjugated NPs.

In tumor pathogenesis, multiple factors such as increased metabolic function, decreased oxygen supply or hypoxia, and depletion of glucose triggers the formation of new blood vessels, a process also known as angiogenesis (328-330). Angiogenesis plays an important role in the proliferation, migration and maintenance of healthy tissues. Imbalance between pro-angiogenic and physiological anti-angiogenic signaling leads to the formation of the tumor vessels which are heterogeneous, immature and leakier than normal vessels (330-333). This leakiness is present because of the impaired recruitment of pericytes which are support cells of normal vessels (334). The intercellular openings between endothelial cells become larger than normal transcellular fenestrations. There is a significant heterogeneity in pore size within and between tumor types with nominal size range between 100 nm to 2 μ m (51, 335, 336). The physical pressure arising from cancer cell proliferation causes compression of the intratumoral lymphatics which leads to poor lymphatic drainage or clearance. As a consequence, NPs which are smaller than the fenestrations can extravasate to the surrounding tumor tissues and entrapped in the tumor. This phenomenon is known as enhanced permeability and retention (EPR) effect. EPR effect is also known as passive targeting which has also been exploited by various drug delivery systems including NPs (50). Unlike passive targeting by EPR effect, active targeting utilizes peripherally conjugated targeting moieties such as monoclonal antibodies, proteins, peptides, vitamins or aptamers to direct drug loaded NPs to the specific tumor tissues (337). Targeting ligands can be attached to the surface of NPs by covalent and non-covalent conjugation chemistry techniques (152). Covalent coupling

techniques employ various covalent linkages such as thioether (338), disulfide (339), carboxamide (340), amide (341), and hydrazine bonds (342) to bind ligands to NPs. Non-covalent coupling method used to bind ligands to NPs include simple adsorption. However, the non-covalent method has limitations such as low ligand binding efficiency, incorrect orientation of ligands, difficulty in controlling amount of ligands, and detachment of ligands *in vivo* (343).

Most NSCLC tumors (50-90%) overexpress EGFR or HER1 which are associated with tumor cell proliferation, metastasis, and angiogenesis. Small molecule EGFR tyrosine kinase inhibitors (e.g. erlotinib and gefitinib) inhibit autophosphorylation by binding to the ATP binding site of the intracellular tyrosine kinase domains of EGFR. Alternatively, therapeutic EGFR directed monoclonal antibodies such as Cetuximab (marketed as Erbitux®, Bristol-Myers Squibb company) were used to prevent ligand binding to the extracellular domain of the receptor(344-346). EGFR specific monoclonal antibodies (mAb) were investigated as the targeting ligand in nanoparticulate drug delivery systems in NSCLC (347-353). Whole mAbs (molecular weight 150 kDa) which are immunoglobulins (IgG) have two identical heavy (H) chains and two identical light (L) chains of amino acids held together by disulfide bridges between the cysteine residues (354). The mAbs conjugated onto NPs have several limitations such as shorter circulation half-life because of the large hydrodynamic size of mAbs increases non-specific uptake by RES. Large hydrodynamic size of mAbs also limit both intratumoral uptake and homogeneous distribution in the tumor, thus adversely affecting pharmacokinetic properties of mAb-conjugated NPs (355). In addition, binding orientation is important for maximizing the functionality of mAbs for targeting (356). However, non-specific conjugation of nanoparticle to mAb via amide bond may hinder the binding sites of mAb (357). Antibody fragments can penetrate tissue more rapidly than whole mAbs due to their smaller size than mAbs (358, 359). Antibody fragments such as single chain variable fragments (scFv), diabodies, and nanobodies have been used to replace mAbs (360, 361). Peng *et al* reported a significant increase in intracellular cisplatin concentration and enhanced antitumor efficacy of the single-chain variable fragment (ScFv) EGFR conjugated heparin NPs (362). A ScFvEGFR functionalized quantum dots (QDs) were reported to bind and internalize by EGFR-expressing cancer cells (363).

Half-antibodies (hAb) or reduced IgG were synthesized by TCEP induced cleavage of the disulfide bridges in the hinge region between two heavy chains of mAb (94, 364). The hAbs possess intact binding site and retain their receptor binding affinity (364, 365). NPs can be conjugated through the readily available thiol functional groups that are present in the constant region of hAb (94, 365). The other advantages compared to mAbs include relatively smaller hydrodynamic size, longer circulation half-life, and rapid extravasation to tumor tissues. For the present study, ETB-CSLAHNPs were conjugated to anti-EGFR hAb *via* thioether bonds between maleimide terminated CSLAHNPs and thiol groups of hAb. The objective of this study was to prepare and characterize prototype anti-EGFR hAb-conjugated ETB-CSLAHNPs for the purpose of active targeting to NSCLC.

Experimental Section

Materials

Bovine serum albumin (BSA, Cohn fraction V, lyophilized powder, MW~66000, purity 95-99 %), glutaraldehyde solution (25 % in water), 2-mercaptoethanesulfonic acid sodium salt (MESNA), sodium chloride, and Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) were obtained from Sigma-Aldrich (St. Louis, MO). Erlotinib hydrochloride (purity 99 %) was obtained from LC Laboratories (Woburn, MA). The lipids used in this work were all of research grade. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N (poly(ethylene glycol)2000) [DSPE-PEG(2000)], and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(maleimide poly(ethylene glycol)2000) [DSPE-PEG2000-maleimide] were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was purchased from Sigma (St. Louis, MO). High performance liquid chromatography (HPLC) grade water, ethanol, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). HPLC grade chloroform and 1N sodium hydroxide solution were purchased from Acros Organics (Morris Plains, NJ). Mouse monoclonal EGFR antibody (D-8) was obtained from Santa Cruz (Dallas, TX). Tris(2-carboxyethyl)phosphine hydrochloride [TCEP] was supplied by Thermo Scientific (Rockford, IL). All other chemicals and reagents were of analytical grade and used without further purification or characterization.

Preparation of maleimide terminated ETB-CSLAHNPs

Maleimide terminated ETB-CSLAHNPs were prepared and purified using the method previously reported in Chapter 2 and 3 with the replacement of 1 mol % DSPE-PEG2000 with DSPE-PEG2000-maleimide.

Assay of maleimide

Maleimide content of ETB-CSLAHNPs after incorporation of DSPE-PEG2000-maleimide was quantified using the modified Ellman's assay (366). Briefly, a standard curve was prepared by adding the Ellman's reagents to different concentrations of a water soluble thiol MESNA in 100 mM PBS pH 8. The absorbance was read at 412 nm using a UV-visible spectrophotometer and thiol concentration was quantified from the standard curve. An aliquot of maleimide terminated ETB-CSLAHNPs in PBS was reacted with excess molar concentration of MESNA for 5 min at room temperature, followed by addition of Ellman's reagent and absorbance was read at 412 nm. The concentration of maleimide was calculated by subtracting unreacted MESNA from initial concentration of MESNA added.

Synthesis of anti-EGFR half antibody

For antibody conjugation, anti-EGFR half-antibody (hAb) were synthesized from anti-EGFR intact monoclonal antibody (mAb) by reducing the disulfide linkages between the heavy chains of mAb (94). Briefly, mAb in 1xPBS (50 µg/mL) was mixed with 50:1 molar ratio of [*tris*(2-carboxyethyl)phosphine] (TCEP) to mAb and incubated for 1h at room temperature(364). The samples were passed through Amicon® Ultra-0.4 filters MWCO 100 kDa, followed by 50 kDa. The sample retained in 50 kDa was collected. Total cell proteins from each sample (25-50 µg) were added to Laemmli 5x loading buffer and incubated at 37°C for 10 min. The samples were loaded into the wells of 4-15 % SDS-PAGE gels immersed in running buffer (tris-glycine/SDS) and resolved at 150V for 45 min using standard molecular weight ladder. Finally, the gel was stained with water soluble colloidal Coomassie™ blue stain for 12 h. A calibration curve was constructed using standard concentrations of BSA resolved by SDS-PAGE as described above. The Coomassie-stained gels were used for densitometric measurements using ImageJ 1.39u software (National Institutes of Health, Bethesda, MD). Using a calibration curve, the densitometric units of the samples were converted into concentrations.

Preparation of half-antibody conjugated ETB-CSLAHNPs

The molar ratio of hAb to DSPE-PEG2000-maleimide used was 8.6 nM/µM as reported by Meel *et al* (367). CSLAHNPs with maleimide moieties (1 mL) were incubated with selectively reduced anti-EGFR hAb (1mL) for 4h at 4°C. The samples were assayed for maleimide content by modified Ellman's assay as reported previously. The unconjugated antibodies, TCEP and other small molecular weight species were removed by spinning the dispersion in Sepharose Cl-4B packed gel filtration column. To confirm the presence of hAb on targeted CSLAHNPs, the samples were resolved by SDS-PAGE and hAb concentration was determined by the method described above.

Characterization of half-antibody conjugated ETB-CSLAHNPs

The mean diameter, polydispersity index (PDI), zeta potential, morphology, drug loading, drug release, and *in vitro* serum stability of the targeted CSLAHNPs were determined using the methods reported in Chapter 2 and 3.

Results and Discussion

The schematic diagram of the preparation of targeted ETB-CSLAHNPs was shown in **Figure 4-1**. To prepare targeted ETB-CSLAHNPs, anti-EGFR half-antibody (hAb) was conjugated to maleimide-terminated ETB-CSLAHNPs. The preparation of hAb from monoclonal antibody (mAb) via disulfide bond reduction method was the critical step in the preparation of targeted hAb-ETB-CSLAHNPs. A number of protein disulfide reducing agents are available such as β-mercaptoethanol (BME) (368), cysteine,

Figure 4-1. Schematic diagram of the preparation of anti-EGFR half antibody (hAb) conjugated ETB-CSLAHNPs

Anti-EGFR monoclonal antibody (mAb) was selectively reduced to half-antibody (hAb) with thiol groups. Maleimide-PEG-DSPE incorporated ETB-CSLAHNPs were conjugated with hAbs to produce hAb-ETB-CSLAHNPs.

dithiothreitol (DTT) (369), tris(2-carboxyethyl)phosphine (TCEP). In this study, TCEP was used as the reducing agent because it is stable, specific, and effective at wide range of pH (370). Anti-EGFR mAb was selectively reduced by using TCEP which breaks the disulfide bonds between the two heavy chains of the mAb to form hAb or reduced IgG. The extent of mAb reduction was controlled by TCEP concentration and duration of reaction (94, 364). Normally, the reduction products include half-antibody or hAb fragments (~75kDa), heavy chain fragments (~ 50 kDa), light chain fragments (~25 kDa) along with unreacted mAb (~ 150 kDa) (371). Various molar ratios of mAb: TCEP were incubated at room temperature for 1 h (364). The samples were resolved in SDS-PAGE and visualized by staining with CoomassieTM blue (**Figure 4-2**). It was observed that 1:100 or higher molar ratio of mAb:TCEP completely cleaved to heavy-chain and light-chain fragments. The optimized molar ratio of 1:50 of mAb:TCEP maximized the yield of hAb and minimize the production of non-targeting fragments.

Maleimide-terminated ETB-CSLAHNPs (Mal-ETB-CSLAHNPs) were prepared by the previously described two-step method using 1 mole % DSPE-PEG2000-maleimide. The final composition of the lipids used was DPPC, cholesterol, DSPE-PEG2000, and DSPE-PEG2000-maleimide at a molar ratio of 60:30:9:1 respectively. Since the incorporation of lipids in the Mal-ETB-CSLAHNPs was not 100%, the molar concentration of maleimide groups on the particles were determined by modified Ellman's assay (366). The Ellman's assay showed actual amount of DSPE-PEG2000-maleimide in CSLAHNPs was ~ 30-40% of the theoretical amount added. The ratio of hAb to DSPE-PEG2000-maleimide was kept at 8.6 nM/ μ M of DSPE-PEG 2000-maleimide as reported previously (367). Based on the theoretical assumptions, it was estimated to be ~ 30 hAb molecules per CSLAHNPs (372). The anti-EGFR mAbs were reduced by TCEP at 1:50 molar ratio for 1 h to produce hAbs with readily available sulfahydryl groups (-SH). Mal-ETB-CSLAHNPs and hAbs were incubated together at 4°C overnight to obtain hAb-conjugated ETB-CSLAHNPs or targeted CSLAHNPs (hAb-ETB-CSLAHNPs). The conjugation of hAbs and CSLAHNPs were accomplished through the formation of thioether linkages between sulfahydryl groups and maleimide groups present on the distal end of PEG chains on the Mal-ETB-CSLAHNPs. To confirm that hAb was successfully conjugated to ETB-CSLAHNPs, samples were run through SDS-PAGE and visualized by staining in comparison with standard molecular weight ladder (**Figure 4-2**). The physicochemical properties of hAb-ETB-CSLAHNPs were reported in **Table 4-1**. Targeted hAb-ETB-CSLAHNPs were ~ 200 nm in mean hydrodynamic size with PDI ~ 0.13, zeta potential ~ -13mV, DL ~ 2 % w/w, and DEE ~ 31% w/w.

The TEM image of hAb-ETB-CSLAHNPs was shown in **Figure 4-3**. It showed that targeted CSLAHNPs were spherical in shape with moderate degree of polydispersity. The conjugation of hAb onto ETB-CSLAHNPs did not significantly change the morphology and shape of the particles compared to ETB-CSLAHNPs without hAb (as shown in Chapter 3).

The drug release data expressed as percentage of drug released from hAb-ETB-CSLAHNPs in two dissolution media (PBS pH 7.4 and acetate buffer pH 5.2) as a

Figure 4-2. Analysis of proteins by SDS/PAGE with coomassie staining

Lane 1 represents ETB-CSLAHNP without targeting half-antibody (hAb) which did not show any protein band. Lane 2 represents ETB-CSLAHNPs conjugated with hAb which showed a distinct band at ~75 kDa. Lane 3 represents TCEP reduced mAb at 1:50 molar ratio of mAb: TCEP. It showed three distinct bands at ~75 kDa, 45 kDa, and 25kDa. Lane 4 represents anti-EGFR mAb which showed a sharp band at ~150 kDa. The rightmost lane represents a standard molecular weight proteins marker.

Table 4-1. Physicochemical properties of targeted CSLAHNPs

Batch	Mean Size (nm)	PDI	Zeta potential (mV)	DL (% w/w)	DEE (% w/w)
Mal-ETB-CSLAHNPs	196 ±5	0.11±0.01	-19.1±5.1	2.12±0.3	31.95±3.34
hAb-ETB-CSLAHNPs	199 ±7	0.13±0.02	-12.6±4.1	2.01±0.2	30.95±4.37

The data were presented as mean ± SD (n=3). Mal-ETB-CSLAHNPs represent 5 mol % DSPE-PEG2000-maleimide incorporated ETB-CSLAHNPs. hAb-ETB-CSLAHNPs represent anti-EGFR half-antibody (hAb) conjugated ETB-CSLAHNPs.

Figure 4-3. TEM images of targeted hAb-ETB-CSLAHNPs

Scale bar represents 100 nm. The sample was stained with 1% sodium silicotungstate. The particles showed smooth and spherical shapes with moderate degree of polydispersity. The lipid shell appeared as a dark corona around each particle.

function of time (**Figure 4-4**). The hAb-conjugated CSLAHNPs showed similar release profiles in pH 7.4 and pH 5.2 compared to ETB-CSLAHNPs without targeting antibody (as shown in chapter 3). The encapsulated ETB was released from the hAb-ETB-CSLAHNPs at a sustained rate over 96 h in PBS pH 7.4 with a biphasic release pattern. In acetate buffer pH 5.2, the drug release rate was faster compared to PBS pH 7.4, indicating pH sensitive release of hAb-ETB-CSLAHNPs (**Figure 4-4**).

NP-serum protein interactions are related to the particular physicochemical characteristics of the particles, such as their colloidal stability, and this significantly influences the subsequent NP-cell interaction. The colloidal stability of hAb-ETB-CSLAHNPs in 50 % fetal bovine serum showed by measuring absorbance at 560nm for various incubation time points (**Figure 4-5**). There was no perceptible change in absorbance values for up to 6 h. After prolonged incubation for 24 h, the absorbance value increased indicating aggregation due to the adsorption of serum proteins. Lipid coating around polymeric core particles also showed enhanced serum stability compared to bare PNPs (373). Surface charge was identified as the critical parameter which influenced the colloidal stability of NPs. It was reported that positively charged NPs displayed a significantly lower colloidal stability than neutral and negatively charged particles (374). The targeted CSLAHNPs possessed negative zeta potential which increased colloidal stability by electrostatic repulsive forces. In addition, the presence of PEG chains on the particle surface provided increased colloidal stability via steric repulsion by reducing serum protein adsorption (375).

Summary and Conclusion

Targeted hAb-ETB-CSLAHNPs were prepared by the maleimide-thiol conjugation reaction after incorporating 1 mol % DSPE-PEG2000-maleimide in ETB-CSLAHNPs during preparation. Anti-EGFR mouse monoclonal antibody (mAb) was reduced to half-antibodies (hAb) using TCEP at 50:1 molar ratio of TCEP:mAb and coupled to maleimide-terminated ETB-CSLAHNPs. Targeted hAb-ETB-CSLAHNPs were ~ 200 nm in hydrodynamic mean size with PDI ~ 0.13, zeta potential ~ -13 mV, DL ~ 2 % w/w, and DEE ~ 31 % w/w. TEM image showed smooth and spherical particles with moderate degree of polydispersity. Drug release from hAb-ETB-CSLAHNPs occurred in a sustained manner up to 72-96 h with a biphasic release pattern in pH 7.4. A faster rate of drug release was observed in acidic pH of 5.2. The hAb-ETB-CSLAHNPs were stable in 50% serum for up to 6 h. Therefore, the hAb-ETB-CSLAHNPs were used for the cell-based assays.

Figure 4-4. *In vitro* drug release profiles of targeted hAb-ETB-CSLAHNPs in pH 7.4 and acetate buffer pH 5.2 at 37°C

The data were presented as mean $\pm$ SD (n=3). Sustained release of drug from CSLAHNPs was observed for about 72-96h. Drug release rate was faster in pH 5.2 compared to pH 7.4.

Figure 4-5. *In vitro* stability of targeted hAb-ETB-CSLAHNPs in 50% fetal bovine serum at 37°C

The data were presented as mean $\pm$ SD (n=3). Particles retained their physicochemical properties without aggregation for a prolonged period of time.

CHAPTER 5. *IN VITRO* UPTAKE, TRAFFICKING, AND EFFICACY OF TARGETED AND UNTARGETED ERLOTINIB LOADED CORE SHELL TYPE LIPID ALBUMIN HYBRID NANOPARTICLES IN NSCLC CELLS

Introduction

A detailed understanding of the involved processes of NPs to cell interaction is an important aspect for developing NPs for anticancer drug delivery (376, 377). Cell membrane acts as a barrier impermeable to many large particles while allowing very small particles of size 10-30 nm (378, 379). One of the mechanisms to overcome the barrier for larger particles is known as endocytosis. It is a process utilized by viruses when they invade cells. Endocytosis has been described as the uptake of particulate matter such as proteins, nutrients, cell debris, and foreign cells via formation of invagination of the cell membranes (380). Phagocytosis is a type of endocytosis in which cells (e.g. macrophages, monocytes, and neutrophils) engulf larger particles (e.g. bacteria, cell debris) of diameter between 0.5 to 10 μm as a response of body's immune defense. In addition to phagocytosis, cells also utilize the pinocytosis process for the uptake of soluble substances. There are two major pinocytic mechanisms currently known: micropinocytosis and receptor-mediated endocytosis (RME). Macropinocytosis is the process of uptake of particles *via* formation of larger vesicles (0.3 – 5 μm) mediated by membrane ruffling driven by actin cytoskeleton (381). There are different pathways observed in RME: clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin- and caveolin-independent endocytosis. Clathrin-mediated endocytosis is the uptake of particles through the formation of clathrin coated pit. The process involves five stages: nucleation, cargo selection, clathrin coat assembly, vesicle scission, and uncoating/clathrin component recycling. The size of the clathrin-coated pits varies from species to species in the range of 15 - 200 nm (382). Caveolae mediated endocytosis involves uptake of particles *via* the formation of invaginated, flask-shaped plasma membrane domains, known as caveolae. These vesicles are typically 50-80 nm in diameter and are enriched with cholesterol and sphingolipids. After internalization, caveolae- or lipid raft-derived vesicles travel to caveosomes. Caveosomes have neutral pH compared to endosomes which have an acidic pH. In caveosomes, internalized cargo could reside, sorted to the Golgi complex, or to the endoplasmic reticulum. In addition to active endocytotic processes, NPs may also enter the cells by non-endocytotic mechanisms such as diffusion or active transport.

NPs interact with biomolecules including proteins, carbohydrates, and lipids present in the extracellular fluids prior to association with the cell membrane. A protein corona forms around the NPs and subsequently triggers activation of cell surface receptors (383). Plasma protein such as immunoglobulin binding caused opsonization and receptor-mediated phagocytosis of carboxyl-functionalized NPs (264). Cellular uptake and internalization of NPs are strongly influenced by physico-chemical properties of NPs including size (384, 385), shape (386, 387), surface charge (388, 389), surface functional groups (390) and hydrophilicity (391).

Apart from quality, the biological safety and efficacy of nanomedicines have to be fully investigated to comply with the rules and regulations set forth by regulatory agencies such as Food and Drug Administration. For the biological testing of nanomedicines, cell culture models have been widely used as an exceptionally powerful and economic tool before moving to expensive *in vivo* models (392). Cell based models offer unique platform to test the efficacy and safety of nanomedicines under controlled and reproducible conditions (393). In addition, a number of experimental variables can be easily manipulated to simulate the *in vivo* conditions (394).

The objective of this work was to investigate the cellular binding, uptake and internalization of fluorescently labeled ETB-CSLAHNPs (untargeted) and hAb-ETB-CSLAHNPs (targeted) in two human lung adenocarcinoma cells (A549 and HCC827). In addition, *in vitro* efficacy of CSLAHNPs was determined by cell viability assay and clonogenic assay. Finally, the expression levels of biomarker (p-EGFR) with and without treatment of CSLAHNPs were assessed by western blot.

Experimental Section

Materials

Bovine serum albumin (BSA, Cohn fraction V, lyophilized powder, MW~66000, purity 95-99%), and cholesterol were obtained from Sigma-Aldrich (St. Louis, MO). The lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N (poly(ethylene glycol)2000) [DSPE-PEG2000], 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(maleimide poly(ethylene glycol)2000) [maleimide DSPE-PEG2000], and 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). Mouse monoclonal EGFR antibody (D-8) was obtained from Santa Cruz (Dallas, TX). Tris(2-carboxyethyl)phosphine hydrochloride [TCEP] was supplied by Thermo Scientific (Rockford, IL). CellTiter-Glo[®] Luminescent cell viability assay substrate and buffer was purchased from Promega (Madison, WI). High performance liquid chromatography (HPLC) grade water, ethanol, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). All other chemicals and reagents were of analytical grade and used without further purification or characterization.

Preparation of fluorescently labeled CSLAHNPs for imaging

For imaging cellular uptake and intracellular localization, fluorescently labeled ETB-CSLAHNPs (untargeted) and hAb-ETB-CSLAHNPs (targeted) were prepared and purified using the methods reported in Chapter 3 and Chapter 4 respectively with the replacement of 1 mol % DPPC with NBD-PC, a fluorescent phospholipid (Ex/Em 460/530 nm).

Preparation of CSLAHNPs

For *in vitro* cellular viability, colony-forming assay, and western blots, ETB-CSLAHNPs (untargeted) and hAb-ETB-CSLAHNPs (targeted) were prepared and purified using the methods reported in Chapter 3 and Chapter 4 respectively.

Culture of human lung cancer cell lines

Two cell lines, A549 (human lung adenocarcinoma; ATCC[®] CCL-185[™], Manassas, VA, USA) and HCC827 (human lung adenocarcinoma; ATCC[®] CRL-2868[™], Manassas, VA, USA) were used for this project. HCC827 cells have an acquired mutation in the EGFR tyrosine kinase domain (E746 - A750 deletion).

A549 cells were cultured in Ham's F12K (Kaighn's) medium containing 10% foetal bovine serum (FBS), 1% penicillin streptomycin (Penstrep) (all purchased from ATCC, VA, USA). HCC827 cells were cultured in RPMI 1640 medium containing 10% FBS, 1% Penstrep. Cells were grown under standard conditions (37°C and 5% CO₂) to reach a confluency of 70% to 80% before being subjected to any further experimentation.

***In vitro* cellular uptake of fluorescently labeled CSLAHNPs**

To study the cellular uptake of fluorescently labeled CSLAHNPs, two techniques were utilized as given below.

Visualization and qualitative uptake of CSLAHNPs were studied by using confocal laser scanning microscopy (CLSM) technique (392). Cells were seeded in Lab-Tek[®] II chamber slides (4 wells) at a density of 5×10^4 cells per well and incubated at 37°C, 5 % CO₂ in complete growth medium until 80-90 % confluence. Appropriate volume (50-100 μ L) of fluorescent CSLAHNPs were added in each well and incubated for 0-4 h. Following the incubation, cells were washed with 1x PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. Following fixation, cells were washed with 1X PBS thrice 3 min each time. The upper chamber was separated from the slide. The cells were counterstained with a drop of VECTASHIELD[®] mounting medium containing DAPI to visualize nuclei. A coverslip was placed over the slide containing fixed cells. The cover slip was immobilized by placing clear nail polish around the edges. Finally, the slides were examined on a Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss SMT Inc., USA) by using a Plan-Apochromat 60 $\times$ /1.40 na Oil DIC objective. Images were processed using the Zeiss LSM Zen software 2010.

For quantitative estimation of cellular uptake, flow cytometry (FCM) was used (395). Cells were seeded into 6-well plates at a density of 5×10^4 cells per well in 2 mL of complete medium and cultured at 37°C in a 5 % CO₂ humidified atmosphere until 80-90 % confluence. The growth medium was replenished, followed by addition of appropriate volume of fluorescently labeled CSLAHNPs. The cells were incubated for 0-4 h at 37°C,

followed by rinsing with ice-cold 1x PBS three times. The cells were detached by trypsinized, centrifuged at 130 g for 7 min and washed with 1x PBS. The mean fluorescence intensity (MFI) was determined using a BD Accuri™ C6 flow cytometer (Becton Dickinson Inc., NJ, USA) with FITC channel. Generally 10,000 events were acquired per sample, and samples were run in triplicates. Data were analyzed using BD Accuri™ CFlow Plus analysis software.

Cell association study of fluorescently labeled CSLAHNPs

To determine the target binding specificity of hAb-ETB-CSLAHNPs on EGFR cell surface receptors, cell association studies at 4°C were performed as reported by Oliveira *et al* (396). Cells at a density of (1×10^5) were incubated with fluorescently labeled CSLAHNPs (untargeted and targeted) in the dark for 1h at 4°C at a final concentration of 0.05-10 μ M. Thereafter, cells were washed three times with 1% BSA in 1X PBS and the mean fluorescence intensity (MFI) was determined using a BD Accuri™ C6 flow cytometer (Becton Dickinson Inc., NJ, USA) with FITC channel. Generally 10,000 events were acquired per sample, and samples were run in triplicates. Data were analyzed using BD Accuri™ CFlow Plus analysis software. The cell association of targeted and untargeted CSLAHNPs was also conducted by CLSM as described under the section “*In vitro* cellular uptake of CSLAHNPs”.

Cellular uptake mechanism of CSLAHNPs

To elucidate potential cellular uptake pathways of CSLAHNPs, cells were pre-treated with following endocytosis inhibitors at a concentration that were not toxic to cells. Genistein (200 μ M) (397), chlorpromazine (10 μ g/mL)(398), amiloride (10 mM) and methyl- β -cyclodextrin (10 mM)(399) were added to block caveola-mediated endocytosis, clathrin-mediated endocytosis, micropinocytosis, lipid rafts and lipid-raft-mediated endocytosis respectively. Following pre-incubation with inhibitors for 1h, fluorescently labeled CSLAHNPs were added for further 1 h. Subsequently, the cells were washed thrice with 1xPBS and analyzed using a BD Accuri™ C6 flow cytometer (Becton Dickinson Inc., NJ, USA) with FITC channel. Generally 10,000 events were acquired per sample, and samples were run in triplicates. Data were analyzed using BD Accuri™ CFlow Plus analysis software. CSLAHNPs without any inhibitor treatment were used as control, and the mean fluorescence intensity (MFI) of controls was expressed as 100 %.

Intracellular trafficking of CSLAHNPs

Endolysosomal trafficking of CSLAHNPs was studied in cells using confocal laser scanning microscopy (CSLM) and transmission electron microscopy (TEM). For CLSM, cells were grown on Lab-Tek® II 4-chambered slides with complete growth medium until 70-80 % confluence. The cells were replenished with complete growth

medium containing fluorescently labeled CSLAHNPs. LysoTracker[®] deep red (a specific marker for secondary endosome and lysosome) at a final concentration of 50nM was added into the medium 15 min prior to the experimental endpoint. Following the incubation, cells were washed with 1x PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. Following fixation, cells were washed with 1X PBS thrice 3 min each time. The upper chamber was separated from the slide. The cells were counterstained with a drop of VECTASHIELD[®] mounting medium containing DAPI to visualize nuclei. A coverslip was placed over the slide containing fixed cells. The cover slip was immobilized by placing clear nail polish around the edges. Finally, the slides were examined using confocal microscopy (Zeiss LSM 710, Jena, Germany) by using a Plan-Apochromat 60×/1.40 na Oil DIC objective. Images were processed using the Zeiss LSM Zen software 2010.

For TEM, CSLAHNPs loaded with 10% water soluble iron-oxide NPs with carboxyl group (IONPs, size 15 ± 2.5 nm, Ocean NanoTech, Springdale, AR) were prepared by the two-step method described in Chapter 4. Cells were seeded in BioFlex[®] Collagen type I 6-well plates at a density of 1×10^5 cells/mL. After 24 h incubation, cells were treated with 200 µg/mL IONP-loaded CSLAHNPs for 0-4h. The cells were washed with cold 1X PBS for 3 times and fixed in 4 % paraformaldehyde solution in 1x PBS overnight. The cells were rinsed with 0.1M cacodylate buffer, 3 x 20 min at RT on rotator and dehydrated with successive 50, 70, 80, 90, 95, 100 % ethanol for 30 min at RT on rotator. The cells were infiltrated with 1:1 ethanol:Spurr's resin, overnight at RT on rotator, followed by 100 % Spurr's resin for 2 h at RT on rotator. The cells were embedded in an epoxy resin molds with appropriate labels, the plastic was cured in oven for 48 h at 70° C, cut into ultra-thin sections with an ultramicrotome using a diamond knife, and mounted on 150 mesh copper grids. The cell ultrastructures were stained with uranyl acetate and lead citrate and then observed under TEM.

***In vitro* efficacy of CSLAHNPs in NSCLC cells**

The CellTiter-Glo[®] Luminescent cell viability assay was used for the determination of the number of viable cells in culture based on the quantitation of ATP, an indicator of metabolically active cells. The cells were seeded in 96 well opaque (white) plates at a density of 5000 cells per well and incubated for 24 hours at 37°C, 5% CO₂ in complete medium. Then, the culture medium was replenished with 100 µL of CSLAHNPs at different concentrations (0-15 µM) in complete medium and incubated for 24-72 h. The CellTiter-Glo[®] buffer and lyophilized CellTiter-Glo[®] Substrate were equilibrated at room temperature. The substrate was reconstituted with the buffer to form the CellTiter-Glo[®] reagent. The plate contents were equilibrated at room temperature for approximately 30 min. A 100 µL portion of CellTiter-Glo[®] reagent were added to each well and mixed for 2 min on an orbital shaker to induce cell lysis. Control wells containing complete growth medium without cells were prepared to measure the background luminescence. The plates were equilibrated at room temperature for 10 min to stabilize the luminescent signal. Finally, luminescence was recorded using SpectraMax[®] M2e microplate reader (Molecular devices, Sunnyvale, CA).

Colony-formation assay

Colony formation assay, also known as clonogenic assay were performed by the previously reported method (400). The cells were plated at a density of 500 to 1000 cells on 6-well tissue culture treated plates and allowed to attach for 24 h. After that, cells were treated with CSLAHNPs or controls and allowed to grow for 10-14 days. The cell colonies were washed with 1x PBS thrice, followed by fixing with -20°C methanol in freezer for 5-15 min. The colonies were washed with 1x PBS thrice and stained with hematoxylin. The photographs were taken using GelDoc-it imaging system (UVP, Upland, CA). The efficacy of the CSLAHNPs was determined by counting the number of colonies and comparing with controls.

Expression of phosphorylated EGFR

Expression of phosphorylated EGFR was investigated by western blotting. Cells were seeded into 6-well plates at a concentration of 2×10^5 cells per well. When 90-95% confluency reached, cells were treated with ETB/ ETB-CSLAHNPs/hAb-ETB-CSLAHNPs at the corresponding IC50 values and incubated for 6 h, followed by stimulation using 60 ng/mL EGF for 15 min. Cells were washed with ice cold 1X PBS thrice and lysed with 0.2% Triton X-100 in 1X PBS on the shaker at 4°C for 1 h. The lysates were scraped out, placed in Eppendorf tubes and centrifuged at 13000 rpm for 15 min. Protein contents in the lysates were estimated by Pierce BCA protein assay kit by optical absorbance method using BSA as the standard. Total cell proteins from each sample (25-50 µg) were added to Laemmli 5x loading buffer and incubated at 37°C for 10 min. The samples were loaded into the wells of 4-15% SDS-PAGE gels (Mini-PROTEAN® TGX Gels, Bio-Rad, Hercules, CA) immersed in running buffer (tris-glycine/SDS) and resolved at 150V for 45 min using standard molecular weight ladder. The transfer stacks were prepared with the order sponge-3 filter papers-PVDF membrane-gel-3 filter papers-sponge. The PVDF membrane was soaked with methanol before placing in the transfer stack. The gel bands were transferred to PVDF membrane at 4°C, 90 V for 1.5 h, followed by blocking in blocking buffer (5% non-fat milk in TBST) and probed using appropriate dilutions of primary monoclonal antibodies directed against phospho-specific EGFR (p-EGFR) and β -actin as control at 4°C overnight. The membrane was washed thrice with TBST 5 min each, followed by recommended dilution of labeled secondary antibody in 5 % blocking buffer in TBST at room temperature for 1 h. The membrane was washed thrice with TBST 5 min each. Immunoreactive bands were detected by enhanced chemiluminescence reagents (Amersham Biosciences).

Statistical analysis

Student's t-test (two tailed) was performed to compare the mean values of different groups; p values less than 0.05 were considered to be significant. All the results are represented as mean $\pm$ SD, with n equaling the number of experiments.

Results and Discussion

EGFR is a tumor specific receptor overexpressed in various NSCLCs (401). Two representative human lung cell lines, A549 and HCC827, were used to test the cellular uptake and efficacy of targeted and untargeted CSLAHNPs. A549 is a lung carcinoma cell line which expresses wild type EGFR. Constitutive upregulation of autocrine/paracrine secretion of EGFR ligands, particularly ARG, results in an increase in active phosphorylated-EGFR in A549 cells (402). HCC827 is a human lung adenocarcinoma cell line that has an acquired mutation in the EGFR tyrosine kinase domain (exon 19 deletion or the del E746-A750 mutation) (403). Literature reports support that HCC827 and A549 cells overexpress EGFR (404, 405).

To study the cellular uptake of CSLAHNPs, both confocal laser scanning microscopy (CLSM) and flow cytometry (FCM) techniques were utilized. CLSM visualized the localization of CSLAHNPs in the cellular compartments, while FCM gave quantitative analysis of the mean fluorescence intensity (MFI) of the treated cells. The application of these two techniques in parallel helped to visualize as well as quantify the uptake of CSLAHNPs in human lung adenocarcinoma cells. Both hAb-ETB-CSLAHNPs (targeted) and ETB-CSLAHNPs (untargeted) were labeled with 1 mol % of a fluorescently labeled phospholipid, NBD-PC for visualization and quantification. The physicochemical properties of fluorescent targeted and untargeted CSLAHNPs such as size, PDI and zeta potential were almost similar to the non-fluorescent targeted and untargeted CSLAHNPs (data not reported).

CLSM images of the cellular uptake of CSLAHNPs at two time points were shown in **Figure 5-1** for HCC827 cells and in **Figure 5-2** for A549 cells. Upon excitation at 460 nm, the NBD-PC tagged CSLAHNPs appeared green under CLSM (emission wavelength of NBD-PC at 534 nm). DAPI (Ex/Em 358/461 nm) was used for nuclear staining (blue color). The appearance of green color surrounding the blue colored nucleus indicated that the CSLAHNPs were extensively present in the cytoplasm of the cells. In both cells, significantly higher uptake of targeted and untargeted CSLAHNPs was observed at 3.5 h compared to 0.5 h. Higher uptake was observed with hAb-ETB-CSLAHNPs in HCC827 cells compared to untargeted ETB-CSLAHNPs.

As shown by FCM, NSCLC tumor cells treated with 1 mol % fluorescent NBD-PC lipid loaded CSLAHNPs exhibited a time dependent increase of their mean fluorescence intensity (MFI) which indicated enhanced intracellular uptake of CSLAHNPs. It was significantly different from the control only after 30 min of exposure in both the cells A549 and HCC827 (**Figure 5-3**). Overall, a higher cellular uptake was observed in HCC827 cells compared to A549 cells. Targeted NBD-hAb-ETB-CSLAHNPs were taken up significantly higher in HCC cells after 4h compare to untargeted ETB-CSLAHNPs. As observed by CLSM, CSLAHNPs were found inside the cytoplasm of cells. Observation of treated cultures revealed that CSLAHNPs were almost entirely internalized after 4 h suggesting that the MFI measured by FCM mainly reflected internalized CSLAHNPs. Therefore, the results of both FCM and CLSM confirmed the uptake of CSLAHNPs.

Figure 5-1. Confocal microscopy images of CSLAHNPs uptake in HCC827 cells after 0.5 h (A) and 3.5 h (B)

DAPI stained nucleus, NBD represents fluorescent NBD-PC loaded CSLAHNPs.

Figure 5-2. Confocal microscopy images of CSLAHNPs uptake in A549 cells

(A) 0.5 h and (B) 3.5 h . DAPI stained nucleus, NBD represents fluorescent NBD-PC loaded CSLAHNPs.

Figure 5-3. Flow cytometric analysis of cellular uptake of CSLAHNPs in HCC827 cells

(A) and A549 cells (B). The data were presented as mean $\pm$ SD (n=3). * represents p-value of <0.05.

Cell association studies at 4°C were performed to determine whether hAb-ETB-CSLAHNPs could specifically interact with EGFR expressed on the cell surface. For comparison, control sets were run at 37°C. After 1h incubation at 4°C, cell associations of fluorescent CSLAHNPs were determined by measuring MFI using FCM (**Figure 5-4**). A549 cells showed no significant difference in MFI between targeted and untargeted CSLAHNPs. As expected, HCC827 cells incubated with targeted CSLAHNPs presented 4 fold higher MFI than untargeted CSLAHNPs. CLSM images confirmed the observed anti-EGFR hAb mediated cell association of targeted CSLAHNPs compared to untargeted particles (**Figure 5-5**). A higher degree of association was observed for both targeted and untargeted in HCC827 cells compared to A549 cells. This could be explained by a higher level of EGFR expression in HCC827 compared to A549. The presence of anti-EGFR hAb on the surface of CSLAHNPs resulted in a specific cell association, and enhanced signal compared to control groups. At 37°C, targeted and untargeted CSLAHNPs were taken up by both the cells.

The process of cellular uptake of NPs via endocytosis pathway can involve clathrin mediated endocytosis, caveolae mediated endocytosis, micropinocytosis, and clathrin/caveolae independent endocytosis (377). The flow cytometry results of the effect of endocytosis inhibitors on the uptake of fluorescently labeled CSLAHNPs in both A549 and HCC827 cells were shown in **Figure 5-6**. Macropinocytosis is a non-specific, ligand independent mechanism which involves formation of large irregular vesicles known as macropinosomes (406). Amiloride is an inhibitor of micropinocytosis by inhibiting Na⁺/H⁺ exchange (407). It had no significant inhibitory effect on the CSLAHNPs uptake compared to control in both A549 and HCC827 cells. Chlorpromazine is known to block assembly of clathrin-coated pit formation at the plasma membrane and causes clathrin lattices to assemble on endosomal membranes (408). The treatment with chlorpromazine did not decrease cellular uptake of CSLAHNPs compared to control group in both A549 and HCC827 cells. Caveolae mediated endocytosis involves the formation of 50-100 nm invaginated plasma membranes known as caveolae (409). Caveolae are rich in caveolin, glycolipids, and cholesterol. Genistein is a specific inhibitor of caveolae-mediated endocytosis (410). It showed a significant inhibition of cellular uptake of CSLAHNPs in both cells. Methyl- β -cyclodextrin was used to block cholesterol depletion and lipid-raft mediated endocytosis (399). It did not inhibit the cellular uptake of CSLAHNPs. Therefore, the results showed that the caveolae-mediated endocytosis played a major role in the cellular uptake of CSLAHNPs in human lung adenocarcinoma cells. One of the major components of CSLAHNPs was the albumin NPs. Albumin NPs were reported to bind to protein on the endothelial cell surface localized on caveolae and activate gp60/caveolae receptor mediated endocytosis (411). Therefore, the literature report confirmed the present finding.

In vitro cellular internalization, localization and intracellular trafficking were investigated by TEM. **Figure 5-7** showed the TEM image of the cells incubated with IONPs-loaded CSLAHNPs for 4h. IONPs were loaded into CSLAHNPs to track them inside the cells. IONPs were observed at or near cell membranes, inside the cytoplasm and also into the endolysosomal vesicles. **Figure 5-8** showed the confocal microscopy images of cells incubated with fluorescently labeled CSLAHNPs for various time

Figure 5-4. Cell association study using flow cytometric analysis of cellular uptake of CSLAHNPs in HCC827 cells and A549 cells at 4°C and 37°C

The data were presented as mean $\pm$ SD (n=3). * represents p-value of <0.05.

HCC827

A549

Figure 5-5. Confocal microscopy images of the cell association of CSLAHNPs after 1 h incubation at 4°C

DAPI stained nucleus, NBD represents fluorescent NBD-PC loaded CSLAHNPs.

Figure 5-6. Effect of endocytosis inhibitors on the cellular uptake of CSLAHNPs using flow cytometric analysis

The data were presented as mean $\pm$ SD (n=3). * represents p-value of <0.05. Inhibitors were treated with cells for 1 h, followed by CSLAHNPs incubation for 1 h.

Figure 5-7. TEM image of a single HCC827 cell after incubation of IONPs-loaded targeted CSLAHNPs

The image revealed intracellular localization of 15 nm iron-oxide nanoparticles (IONPs) loaded targeted hAb-ETB-CSLAHNPs after 4h incubation at 37°C.

Figure 5-8. Confocal microscopy image of a single HCC827 cell after 1 h incubation with fluorescent hAb-ETB-CSLAHNPs

The images showed the endolysosomal localization using colocalization of NBD (green) and lysotracker (red) signals in the merged image. The nucleus was stained with blue color.

intervals. The confocal microscopy revealed that the CSLAHNPs were co-localized with endosomes and lysosomes, which were marked by LysoTracker Deep Red.

After confirmation of the binding and internalization of the hAb-ETB-CSLAHNPs and ETB-CSLAHNPs to deliver ETB to target cells, *in vitro* cellular viability of ETB solution, ETB-CSLAHNPs, and hAb-ETB-CSLAHNPs were tested against both A549 and HCC827 cells using CellTitre Glo® Luminescent cell viability assay. Cells were treated with CSLAHNPs or ETB solution for 72h, followed by the determination of cell viability. The plots of ETB concentration versus *in vitro* cellular viability were shown in **Figure 5-9** for HCC827 cells and in **Figure 5-10** for A549 cells. Overall the dose-response curves showed higher sensitivity to ETB, hAb-ETB-CSLAHNPs, and ETB-CSLAHNPs treatments in HCC827 cells compared to A549 cells. The ETB concentrations were in nanomolar (nM) range for HCC cells where high micromolar (μ M) ETB concentrations were required for A549 cells. This could be explained by EGFR expression in both the cells. HCC827 cells express EGFR at a much higher level due to the presence of acquired mutation in the EGFR tyrosine kinase domain (403). A549 cells express a lower level of total and phosphorylated EGFR compared to HCC827 cells (412). In HCC827 cells, hAb-ETB-CSLAHNPs were more effective compared to ETB-CSLAHNPs and ETB-solution. The IC₅₀ values (the concentration required to kill 50 % of the cells) were ~ 20 nM for hAb-ETB-CSLAHNPs, ~45 nM for ETB-CSLAHNPs and ~51 nM for ETB-solution. The comparison of IC₅₀ values demonstrated that anti-EGFR hAb conjugated CSLAHNPs were about 2.5 folds more efficacious in reducing viability of HCC cells after 72 h compared to ETB-solution. This could be explained by higher cellular uptake of hAb-CSLAHNPs via receptor mediated endocytosis pathways compared to free ETB in solution form. The untargeted particles showed no significant difference in viability of HCC827 cells compared to ETB-solution form due to the absence of targeting ligands in ETB-CSLAHNPs. In contrast, the A549 cells were less sensitive to ETB, hAb-ETB-CSLAHNPs, and ETB-CSLAHNPs treatments as concentrations of ETB to 1 did not result in a significant decrease in proliferation

The clonogenic assay or colony formation assay is an *in vitro* long-term cell survival assay based on the ability of a single cell to grow into a colony consisting of at least 50 cells (400). The assay has been essentially used to test the efficacy and toxicity of anticancer drugs, drug delivery systems and radiation therapies (413). The colony formation assay results are shown in **Figure 5-11**. In HCC827 cells, treatment of ETB-CSLAHNPs and hAb-ETB-CSLAHNPs at a concentration close to IC₅₀ (10 nM) significantly decreased the formation of colonies compared to control groups. The ETB solution form also showed decreased number of colonies at 10 nM concentration but not to the same extent as CSLAHNPs. Control and blank CSLAHNPs showed very high colony counts as expected. In A549 cell, reduced number of colonies was observed after treatment of ETB-CSLAHNPs and hAb-ETB-CSLAHNPs at 5 mM compared to control group and blank CSLAHNPs. The ETB-solution form had significantly higher number of colonies compared to hAb-ETB-CSLAHNPs and ETB-CSLAHNPs groups. Therefore, overall increased efficacy was observed in both untargeted and targeted CSLAHNPs compared to ETB solution and controls.

Figure 5-9. Plot of the *in vitro* HCC827 cell viability as a function of ETB (drug) concentration after 72 h

CellTiter-Glo[®] Luminescent cell viability assay was used. The data were presented as mean $\pm$ SD (n=3). * represents p-value of <0.05. IC₅₀ targeted CSLAHNPs was ~20 nM which was lower than the IC₅₀ of untargeted CSLAHNPs (~45 nM) and ETB-solution (~51 nM).

Figure 5-10. Plot of the *in vitro* A549 cell viability as a function of ETB (drug) concentration after 72 h

CellTiter-Glo[®] Luminescent cell viability assay was used. The data were presented as mean $\pm$ SD (n=3). * represents p-value of < 0.05 . IC₅₀ of targeted and untargeted CSLAHNPs $\sim 5\mu\text{M}$ which was lower than the IC₅₀ of ETB-solution $> 20\mu\text{M}$.

Figure 5-11. Colony formation assay results after in (A) HCC827 cells and (B) A549 cells

ETB dose was 50nM for HCC cells and 5 μ M for A549 cells.

The phosphorylated EGFR (active form) expression by western blotting was showed in **Figure 5-12**. The drug (ETB) binds with the ATP binding site in the intracellular tyrosine kinase domain of EGFR. Thus, it inhibits phosphorylation of EGFR to its active form, thereby preventing downstream signaling cascade leading to cell proliferation and angiogenesis. In HCC827 cells, the treatment of ETB-CSLAHNPs at 50 nM showed significant down regulation of p-EGFR expression compared to control. Treatment with only ETB-solution did not show significant downregulation of p-EGFR compared to control. Blank CSLAHNPs had no effect on p-EGFR expression in HCC827 cells. A549 showed lesser sensitivity to ETB or ETB-CSLAHNPs treatments. At 5 μ M ETB-CSLAHNPs, slight reduction in p-EGFR expression was observed compared to control.

Summary and Conclusion

In summary, cellular uptake, intracellular trafficking, and cellular viability of ETH-CSLAHNPs and targeted hAb-ETB-CSLAHNPs were investigated in A549 and HCC827 cells. For intracellular imaging, 1 mol % of NBD-PC was incorporated into CSLAHNPs. The targeting ability of hAb-ETB-CSLAHNPs was confirmed by cell association study. Confocal microscopy and flow cytometry results showed higher uptake of hAb-ETB-CSLAHNPs and ETB-CSLAHNPs in HCC827 cells compared to A549 cells up to 4 h. Endocytosis inhibitor assay showed major mechanism of particle uptake was *via* caveolae-mediated endocytosis. Significantly higher reduction in cell viability was observed for both targeted and untargeted ETB-CSLAHNPs compared to ETB-solution form in HCC827 compared to A549 cells. Similar results were observed for colony formation assay. Western blot results showed significant downregulation of phosphorylated EGFR after ETB-CSLAHNPs treatment compared to control. The results suggest the potential therapeutic advantages of targeted and untargeted ETB-CSLAHNPs over ETB in solution in EGFR-overexpressing NSCLC.

Figure 5-12. Expression of phosphorylated-EGFR by western blot

(1) ETB-solution, (2) control, (3) hAb-ETB-CSLAHNPs, and (4) blank hAb-CSLAHNPs. ETB dose was 50 nM for HCC cells and 5 μ M for A549 cells. All treatments were incubated with the cells for 6 h. The blots showed higher downregulation of phosphorylated EGFR (active form) after ETB-CSLAHNPs treatment in HCC827 cells.

CHAPTER 6. LYOPHILIZATION OF TARGETED AND UNTARGETED ERLOTINIB LOADED CORE SHELL TYPE LIPID ALBUMIN HYBRID NANOPARTICLES

Introduction

Physical and chemical stability of the colloidal drug delivery systems including CSLAHNPs in aqueous dispersion is a major concern in the development of a drug product with acceptable shelf-life (344). Phospholipids which constitute the shell of the CSLAHNPs can undergo hydrolysis and/or oxidation and lead to degradation when stored as aqueous dispersions (414). As a result, the encapsulated drug tends to leak from the bilayer which surrounds the core of CSLAHNPs (415). Apart from drug leakage, the CSLAHNPs may aggregate or fuse together resulting in an increase in particle size distribution. The albumin core and the targeting antibody conjugated to the CSLAHNPs are also susceptible to chemical and physical degradation pathways, such as oxidation, deamidation, aspartate isomerization, peptide bond hydrolysis and aggregation (416-419). The long-term stability of colloidal drug delivery systems including CSLAHNPs can be improved by various techniques such as freezing-drying, lyophilization, spray drying, and supercritical fluid technology. Lyophilization is one of the preferred methods to improve long-term stability of NPs containing thermosensitive drugs and proteins (170). It can prevent a number of physical and chemical degradation pathways during storage (170). By removing water by lyophilization process, the physicochemical characteristics of CSLAHNPs can be retained with improved storage stability.

Lyophilization is the process of removing water from a frozen sample by sublimation and desorption to increase the shelf-life of materials (420-422). It combines three separate and interdependent processes; freezing, primary drying (sublimation), and secondary drying (desorption). The first step is the freezing which is initiated by cooling the shelves to the desired freezing temperature and holding the temperature to equilibrate and create a solid matrix. The key process parameters for freezing step include freezing temperature, freezing rate and hold time at the freezing temperature. The freezing temperature should be below the glass transition temperature (T_g) of the formulation. A slow cooling rate results in relatively homogenous ice crystal formation. The majority of water is removed in the primary drying by the process of sublimation. There are two critical process parameters; shelf temperature and chamber pressure. The shelf temperature is maintained 2-5°C below T_g of the frozen product to maintain structural integrity of the maximally frozen amorphous phase and prevent collapse. For crystalline materials, product temperature must be kept below the eutectic melting temperature (T_e) of the crystalline component in the formulation to prevent meltback. The chamber pressure is kept below the saturated vapor pressure of the ice at frozen product temperature to provide driving force for sublimation process to occur. The final stage is the secondary drying, the bound unfrozen water is removed via desorption by raising the shelf temperature at ambient or higher values while maintaining low chamber pressure. To characterize the frozen samples, subambient analytical techniques are utilized such as differential scanning calorimetry (DSC) and freeze-drying microscopy (170).

Lyophilization may generate various stresses such as cryo/freezing and lyo(sublimation) stresses which could destabilize NPs. In order to minimize the stresses and subsequent destabilization, excipients are added before the lyophilization process. The excipients include cryoprotectant, lyoprotectant, bulking agents, buffers, tonicity adjusters, and collapse temperature modifiers. Cryoprotectants are used to protect the product from freezing stress while lyoprotectants are added to protect the product from sublimation stress (423). Cryoprotectants stabilize NPs by preferential “exclusion” mechanism (424). The examples of cryoprotectants include polyethylene glycol, glycerol, and dimethylsulfoxide (425). A number of excipients are available as lyoprotectants such as glucose, sucrose, trehalose, mannitol, lactose, sorbitol, glycine, dextran, fructose, maltose, glycerol, polyvinyl alcohol (426). They stabilize the NPs by either “water substitution” or “vitrification” mechanism (427). In vitrification mechanism, the glassy sugar matrix below T_g immobilizes NPs and prevents aggregation.

The specific aim of this study was to develop lyophilized hAb-ETB-CSLAHNPs (targeted) and ETB-CSLAHNPs (untargeted) CSLAHNPs of ETB to improve the shelf-life of the CSLAHNPs during storage. The cryo/lyoprotective ability of sucrose and trehalose at various concentrations during the process of lyophilization was investigated to optimize the better protectant and its concentration. Finally, the stability of liquid dispersion and lyophilized dry powder were evaluated after stored at different storage conditions for various lengths of time.

Experimental Section

Materials

Bovine serum albumin (BSA, Cohn fraction V, lyophilized powder, MW~66000, purity 95-99%), glutaraldehyde solution (25% in water), sucrose, and trehalose were obtained from Sigma-Aldrich (St. Louis, MO). Erlotinib hydrochloride (purity 99%) was obtained from LC Laboratories (Woburn, MA). The lipids used in this work were all of research grade. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N (poly(ethylene glycol)2000) [DSPE-PEG(2000)], and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(maleimide poly(ethylene glycol)2000) [maleimide DSPE-PEG(2000)] were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was purchased from Sigma (St. Louis, MO). High performance liquid chromatography (HPLC) grade water, ethanol, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). HPLC grade chloroform and 1N sodium hydroxide solution were purchased from Acros Organics (Morris Plains, NJ). Mouse monoclonal EGFR antibody (D-8) was obtained from Santa Cruz (Dallas, TX). Tris(2-carboxyethyl)phosphine hydrochloride [TCEP] was supplied by Thermo Scientific (Rockford, IL). All other chemicals and reagents were of analytical grade and used without further purification or characterization.

Preparation of ETB-CSLAHNPs and hAb-ETB-CSLAHNPs

ETB-CSLAHNPs (untargeted) and hAb-ETB-CSLAHNPs (targeted) were prepared by the methods as described in Chapter 3 and Chapter 4.

Determination of T_g

Two lyoprotectants were used; sucrose and trehalose. The effect of lyoprotectant on glass transition temperature (T_g') was monitored by DSC using a Q2000 differential scanning calorimeter equipped with refrigerated cooling system (TA Instruments, New Castle, DE). About 10-20 μ L of the CSLAHNPs and lyoprotectant mixture was placed into T-Zero aluminum pans and hermetically sealed. An empty pan prepared in a similar manner was used as reference. The samples were equilibrated at 25°C and then cooled at 5°C/min to -70°C. After equilibration, the samples were heated at the rate of 10°C/min to 30°C. The T_g' was also determined from these thermograms.

Lyophilization of CSLAHNPs

ETB-CSLAHNPs and hAb-ETB-CSLAHNPs dispersions were mixed with sucrose or trehalose solution. Different weight ratios of CSLAHNPs to lyoprotectant were used (1:1, 1:4, 1:8, and 1:16). The samples (1 mL) were filled into 5cc glass lyo vials. Rubber lyo stoppers placed in the vials with partial opening to allow water vapor escape. The vials containing stoppers were placed into the tray and loaded into the benchtop lyophilizer (VirTis adVantage Plus, SP Scientific, Gardiner, NY). The lyophilization cycle was designed based on the T_g' of maximally freeze-concentrated amorphous phase as determined by DSC. The following parameters were selected: freezing shelf temperature -45°C (duration 6h), primary drying shelf temperature -35°C (duration 12 h), and secondary drying shelf temperature 15°C (duration 6 h), primary drying chamber pressure 100 mTorr, secondary drying chamber pressure 50mTorr. The product temperature, shelf temperature, and condenser temperature were controlled and monitored using Wizard 2.0 software. On completion of the drying cycle, the vials were sealed after filling nitrogen in the headspace of the vials at atmospheric pressure.

Characterization of lyophilized CSLAHNPs

The lyophilized powders were inspected for cake volume and cake appearance including collapse or meltback and skin formation.

The moisture content of the lyophilized cake was determined using Mettler Toledo DL31 Karl Fischer Titrator (Mettler Toledo Inc., Boston, MA). The titrator was calibrated on each day of analysis with a known quantity of water. About 10-20 mg of the lyophilized powder was weighed accurately and added to the titration vessel. The titration was started after allowing one minute for extraction. Triplicate measurements were

recorded. The residual moisture content in the lyophilized cake was expressed in % w/w.

The lyophilized powders were reconstituted with HPLC grade water. The hydrodynamic mean size (Z-avg) of the reconstituted CSLAHNPs was determined using dynamic light scattering using Zetasizer Nano ZS (Malvern Instruments, Westborough, MA) equipped with 4.5 mW diode laser as a source of light operating at 670 nm. The samples were suitably diluted with HPLC grade water. Three independent measurements were performed for each sample. Zeta potential was determined from electrophoretic mobility using samples suitably diluted with HPLC grade water.

Stability evaluation of liquid and lyophilized CSLAHNPs

Short-term stability was assessed for 3 months by after storage of ETB-CSLAHNPs and hAb-ETB-CSLAHNPs (targeted). The different batches of targeted and untargeted CSLAHNPs (aqueous dispersion and lyophilized powder) were stored at two temperatures 2-8°C and 25°C in glass vials for up to 3 months. After predetermined time intervals, liquid samples were characterized for the physicochemical properties including mean size, PDI, zeta potential. For lyophilized batches, powders were reconstituted with water and evaluated for physicochemical properties. The total and free ETB in the CSLAHNPs before and after lyophilization were determined using an HPLC method. Briefly, a centrifugal ultrafiltration device (Amicon[®] Ultra-4, MWCO 100 KD, Millipore, Bedford, MA) was used to separate free drug from the ETB-CSLAHNPs. Drug entrapment efficiency (DEE) and drug loading (DL) of ETB-CSLAHNPs and hAb-ETB-CSLAHNPs were quantified by HPLC method previously described in chapter 3. Finally, the characteristics of CSLAHNPs were compared with that of the initial values.

Results and Discussion

Lyophilization was used to remove water from CSLAHNPs to obtain lyophilized powders for storage stability. The determination of the glass transition temperature (T_g') of the maximally frozen concentrated mixture of the formulation components is a critical parameter which helps to set the freezing and the primary drying temperatures of the lyophilization cycle. To dry the product with retention of the structure of materials, the product is maintained in the solid state below the collapse temperature. The collapse temperature is also known as T_g' for amorphous materials. To determine T_g' , DSC was performed using four different weight ratios of CSLAHNPs and lyoprotectant (1:1, 1:4, 1:8, and 1:16). The DSC thermograms were shown in **Figure 6-1**. The T_g' values decreased with an increase in sucrose weight ratio in the mixture (428). The T_g' value was around -33°C. Therefore, primary drying temperature was set 2-5°C below the T_g' to prevent collapse. For the lyophilization cycle, freezing step was performed at -45°C. The cooling rate was ~ 0.4°C/minute, followed by holding at -45°C for 4 h. The primary drying was performed at -35°C to prevent collapse. The end of primary drying process was determined by the rise in product temperature which matched with the shelf temperature. The total hold time was found to be around 12 h at around -35°C. The

Figure 6-1. DSC thermograms of CSLAHNPs and sucrose mixture

CSLAHNPs to sucrose weight ratio used were (A) 1:0, (B) 1:1, (C) 1:4, (D) 1:8, and (E) 1:16. The T_g' was determined using Universal Analysis 2000 software (TA instruments, New Castle, DE). Blank CSLAHNPs without drug and targeting ligand were used for the DSC studies. T_g' was found to be around -33°C .

chamber pressure was set at 100 mTorr which was below the vapor pressure of ice at sub-zero temperature (423, 429). The difference between the ice vapor pressure at -35°C and the chamber pressure was the driving force for the sublimation process at primary drying phase. For the secondary drying, the temperature was slowly ramped to 15°C and hold for 4 h with maximum vacuum of 50 mTorr. The end of secondary drying was monitored by measuring the moisture content of the lyophilized cake.

Two disaccharides, sucrose and trehalose were used as lyoprotectants in the lyophilization of CSLAHNPs. Four different weight ratios of CSLAHNPs to lyoprotectant (sucrose or trehalose) were lyophilized. The effect of lyoprotectant sucrose on the mean size and PDI of reconstituted CSLAHNPs dispersions were compared to their mean size and PDI before lyophilization (**Figure 6-2**). Sucrose was able to retain the physicochemical characteristics of CSLAHNPs at the selected ratios of CSLAHNPs to lyoprotectant. Trehalose showed slight increase in mean size at 1:1, 1:4 but maintained the mean size at 1:8 and 1:16 (**Figure 6-3**). The possible explanation could be the presence of core albumin which acted a support to prevent the lipid bilayer shell collapse during lyophilization. Albumin NPs were shown to be stable for 6 months in liquid form without affecting physical stability (305, 430). Therefore, the structural integrity of the CSLAHNPs at lower lyoprotectant ratios could be justified. In addition, the presence of PEG chains on the surface of CSLAHNPs provided additional protection during the freezing phase. PEG has been widely used as a cryoprotectant which prevents the NPs aggregation during the freezing phase. Evaluation of the cake properties of the lyophilized powders with trehalose and sucrose revealed that sucrose was better as lyoprotectant than trehalose (**Figure 6-4**). The major limitation of trehalose is its tendency to crystallize during lyophilization (431-433). Crystallization of the lyoprotectant trehalose could alter the structural integrity of CSLAHNPs during lyophilization or after the storage. Therefore, sucrose was selected as the lyoprotectant for further studies. At 1:16 weight ratio of CSLAHNPs to sucrose, the cake volume was equal to the initial fill volume. In addition, the cake was immediately reconstituted with gentle shaking. The mean size, PDI and zeta potential of CSLAHNPs were retained after lyophilization at 1:16 weight ratio of CSLAHNPs to sucrose. At lower ratios, although physicochemical properties of CSLAHNPs were retained, shrinkage of the cake volume was observed. One of the quality control parameters for any lyophilized product is the measurement of the residual water content of the lyophilized cake. Higher moisture content might lead to poor stability and loss of biological activity (434). The low residual moisture content of 0.5-3% w/w was recommended for lyophilized powders containing both small molecular weight drugs and monoclonal antibodies (435). The moisture content of the lyophilized powders of CSLAHNPs containing 16 times higher sucrose content was in the range of $1.85 \pm 0.25\%$ w/w which is acceptable for storage stability. Therefore, 1:16 weight ratio of CSLAHNPs to sucrose was the optimum ratio used to prepare lyophilized CSLAHNPs.

To further evaluate short-term stability, liquid dispersion and lyophilized ETB-CSLAHNPs and hAb-ETB-CSLAHNPs (containing 1:16 weight ratio of CSLAHNPs to sucrose) were stored at 2–8°C (refrigerated conditions) as well as 25°C (accelerated condition) for up to 60 days. Physicochemical properties including mean size, PDI, and

Figure 6-2. Influence of CSLAHNPs to sucrose weight ratio on mean size(A) and polydispersity index (B) of CSLAHNPs before and after lyophilization

The data were presented as mean $\pm$ SD (n=3). Blank CSLAHNPs without drug and targeting ligand were used and indicated as HNPs.

Figure 6-3. Influence of CSLAHNPs to trehalose weight ratio on mean size (A) and polydispersity index (B) of CSLAHNPs before and after lyophilization

The data were presented as mean $\pm$ SD (n=3). Blank CSLAHNPs without drug and targeting ligand were used and indicated as HNPs.

Figure 6-4. Physical appearance of the lyophilized cakes at various weight ratios of CSLAHNPs to lyoprotectant (A) sucrose and (B) trehalose

At 1:16 weight ratio of CSLAHNPs to sucrose, the cake volume was equal to the initial liquid fill volume and the cake appeared uniform.

zeta potential were analyzed immediately after lyophilization and at various time points up to 60 days. Residual moisture content of the formulations was determined directly after freeze drying and after a storage time of 60 days. Directly after the freeze-drying process, the CSLAHNPs showed residual water content $1.85 \pm 0.25\%$. After a storage period of 60 days, the samples stored at $2-8^{\circ}\text{C}$ showed no significant increase in the residual water content. Samples stored at 25°C showed shrinkage and change in appearance of cake after storage for 1-2 weeks. The determination of residual moisture content using Karl Fisher titration method showed an increase in moisture level to 4-5 % w/w after 1-2 weeks. This may be caused by ingress of moisture in the vials from stoppers or inappropriate container-closure integrity (436). The lyophilized samples stored at 25°C showed shrinkage and change in appearance of cake. Therefore, it can be concluded that storage at 25°C showed significant instability of CSLAHNPs. The lyophilized targeted and untargeted CSLAHNPs showed acceptable long-term storage stability at $2-8^{\circ}\text{C}$ with regard to mean size and PDI after reconstitution (**Figure 6-5**). For comparison, liquid CSLAHNPs dispersions were also analyzed for their physicochemical properties at two conditions. At $2-8^{\circ}\text{C}$ storage of the liquid dispersion, the mean size and PDI changed gradually over storage time of 30 days (**Figure 6-6**). The liquid samples stored at 25°C showed aggregation and formation of sediments within 3-5 days of storage. Therefore, the stability study at 25°C was discontinued.

In the case of drug loaded CSLAHNPs a further aspect, which needs consideration, is the drug loading stability after freeze drying. Freeze-drying process may influence the drug loading efficiency of CSLAHNPs since the process might result in drug loss due to change or loss of the particle structures. Therefore, the samples were also investigated for potentially released or leaked drug after reconstitution. After redispersion the resulting particle suspensions were centrifuged through Amicon® ultrafiltration device with MWCO 100 kDa, followed by HPLC analysis of both filtrate and retained particles. **Figure 6-7** showed the influence of storage time on retention of ETB in lyophilized CSLAHNPs. Immediately after lyophilization, the amount of free drug was low and the percent drug retained in the CSLAHNPs was approximately 90 %. The lyophilized CSLAHNPs were able to retain about 80 % of the drug for up to 60 days at $2-8^{\circ}\text{C}$. However, the liquid dispersion stored at $2-8^{\circ}\text{C}$ showed much higher leakage and lower drug retention about 60 % in CSLAHNPs for up to 30 days (**Figure 6-8**). Therefore, it has to be concluded that the freeze-drying process exerted no significant influence on drug loading stability of CSLAHNPs.

Summary and Conclusion

The present study showed that targeted and untargeted ETB-CSLAHNPs can be stabilized both during the lyophilization process as well as for long-term storage using adequate concentrations of suitable excipients. Among the two disaccharides (sucrose and trehalose) used as lyoprotectants, sucrose was the best in preserving the acceptable physicochemical characteristics at 1:16 weight ratio of CSLAHNPs to sucrose. Storage at room temperature (25°C) had a negative influence on both physical and chemical stability of both lyophilized cake and liquid dispersion form of CSLAHNPs. The study also

Figure 6-5. Effect of storage time on the mean size (A) and polydispersity index (B) of the lyophilized CSLAHNPs at 2-8°C

The data were presented as mean $\pm$ SD (n=3).

Figure 6-6. Effect of storage time on the mean size and polydispersity index of the liquid dispersion of CSLAHNPs at 2-8°C

The data were presented as mean $\pm$ SD (n=3).

Figure 6-7. Influence of storage time on the % drug retention in CSLAHNPs at 2-8°C

The data were presented as mean $\pm$ SD (n=3).

Figure 6-8. Influence of storage time on the % drug retention in liquid dispersion of CSLAHNPs at 2-8°C

The data were presented as mean $\pm$ SD (n=3).

showed that the lyophilized form retained the physicochemical properties CSLAHNPs compared to the liquid dispersion after 2-8°C storage condition for 60 days.

CHAPTER 7. SUMMARY AND CONCLUSION

Lung cancer is the leading cause of cancer related death worldwide. Among various histological subtypes, NSCLC is the most prevalent type accounting for 85 to 90 % of all lung cancer cases. One of the main reasons for the poor survival rates among patients with advanced stages of NSCLC is the limited efficacy of traditional chemotherapy along with severe adverse effects of the high doses of cytotoxic anticancer drugs. To overcome the limitations of conventional chemotherapy, molecularly targeted agents were developed to target specific receptors or pathways of tumor cells with higher specificity. For instance, erlotinib was approved for the treatment of locally advanced or metastatic NSCLC after failure of at least one chemotherapy regimen. ETB is a highly selective inhibitor of HER1/ EGFR-TK. However, limitations of oral administration of ETB tablet include several adverse effects/toxicities in cancer patients and development of drug resistance by P-glycoprotein mediated efflux pump or other pathways by NSCLC tumors. Nanoparticulate carriers are attractive for cancer chemotherapy due to their ability to enhance accumulation of anticancer agents at the tumor site, resulting in an increased tumor cell kill efficacy, reduction in total dose and dose-related adverse effects to healthy cells. In addition, NPs often overcome drug resistance to tumors by evading the normal pathways of drug resistance. Recently, core-shell-type lipid-polymer hybrid nanoparticles have emerged as a promising drug delivery platform. This unique platform combines the mechanical advantages of biodegradable polymeric nanoparticles and biomimetic advantages of liposomes. The system provides advantages such as controllable particle size, surface functionality, high drug loading, potential for entrapment of multiple therapeutic agents, tunable drug release profile, and good serum stability. The overall goal of this project was to develop untargeted and anti-EGFR half-antibody conjugated core-shell type lipid-albumin hybrid nanoparticles (CSLAHNPs) for the targeted delivery of ETB in NSCLC cells. The central hypothesis of the project was that CSLAHNPs will improve therapeutic efficacy and thereby reduce toxicities of ETB in NSCLC therapy.

The first aim was to design, prepare and optimize blank CSLAHNPs without drug and targeting antibody as described in Chapter 2. Blank CSLAHNPs were designed and prepared with albumin core and phospholipid bilayer shell by the two step method using bovine serum albumin, DPPC, cholesterol, and DSPE-PEG2000 as the formulation ingredients. All the ingredients used were biodegradable, biocompatible, non-immunogenic or mildly immunogenic and approved by FDA for human use. The formulation and process variables were identified and optimized for the preparation of blank CSLAHNPs. Various factors influenced the physicochemical properties including mean size, polydispersity index, and zeta potential of both ANPs (core) and CSLAHNPs. For the preparation of ANPs (core) by the desolvation method, the optimized variables include albumin concentration of 20 mg/mL, pH of the albumin solution at 8, ethanol addition rate 1 mL/min, volume ratio of water to ethanol 1:2, and glutaraldehyde-to-albumin ratio 0.47% w/w. For the lipid shell, DPPC, cholesterol, and DSPE-PEG2000 were used at a molar ratio of 60:30:10. The critical parameter of the lipid-film hydration method was identified as the weight ratio of lipid-to-ANPs ($W_{\text{Lipid}}/W_{\text{ANPs}}$) during the

optimization of CSLAHNPs. The experimental $W_{\text{Lipid}}/W_{\text{ANPs}}$ was found around 15% w/w (equivalent $W_{\text{DPPC}}/W_{\text{ANPs}} \sim 8\%$ w/w) which was close to the calculated value ($\sim 10\%$ w/w) based on the theoretical assumptions. TEM images showed smooth and spherical particles of both ANPs (core) and CSLAHNPs with some degree of polydispersity. A distinct electron dense corona around periphery of the CSLAHNPs was visible which was absent in ANPs.

Following the preparation of blank CSLAHNPs, the aim of Chapter 3 was to load the drug erlotinib in the CSLAHNPs to prepare ETB loaded CSLAHNPs. The drug binding capacity of albumin in solution was studied and optimized at various pH conditions, durations of incubation, incubation temperatures, and drug-to-albumin molar ratios. The maximum drug binding capacity of albumin was observed at 12h incubation in pH 7.4 at room temperature with drug-to-albumin molar ratio 10:1. ETB-CSLAHNPs were successfully designed, prepared and optimized by the two step method as described in Chapter 2. Two strategies for drug loading in ANPs were studied; incorporation and sorption method. Sorption method provided better drug loading and entrapment efficiency in ETB-ANPs compared to incorporation method. Additional ETB was incorporated in the lipid bilayer to maximize the drug loading and entrapment efficiency of ETB-CSLAHNPs. ETB-CSLAHNPs were 194nm in size with PDI 0.16, zeta potential -15.6mV, yield 83.5% w/w, DL 2.08% w/w, and DEE 32.75% w/w. SEM and TEM images showed smooth and spherical particles of ETB-CSLAHNPs. Drug release from ETB-CSLAHNPs occurred in a sustained manner up to 96h with a biphasic release pattern in pH 7.4. Drug release was faster in acidic pH of 5.2. Drug release kinetics analysis showed a first order release kinetics with non-fickian diffusion mechanism. ETB-CSLAHNPs were stable in serum for up to 6h.

The specific aim of chapter 4 was to prepare and characterize targeted anti-EGFR half-antibody conjugated ETB loaded CSLAHNPs (hAb-ETB-CSLAHNPs). Targeted hAb-conjugated ETB-CSLAHNPs were prepared by maleimide-thiol conjugation reaction after incorporating 1 mol % DSPE-PEG2000-maleimide in ETB-CSLAHNPs during preparation. Anti-EGFR mouse monoclonal antibody (mAb) was reduced to half-antibodies (hAb) using TCEP at 50:1 molar ratio of TCEP: mAb and coupled to maleimide-terminated ETB-CSLAHNPs. Targeted hAb-ETB-CSLAHNPs were ~ 200 nm in mean hydrodynamic size with PDI ~ 0.13 , zeta potential $\sim -13\text{mV}$, DL $\sim 2\%$ w/w, and DEE $\sim 31\%$ w/w. TEM image showed smooth and spherical particles with moderate degree of polydispersity. Drug release from hAb-ETB-CSLAHNPs occurred in a sustained manner up to 96h with a biphasic release pattern in pH 7.4. A faster rate of drug release was observed in acidic pH of 5.2. The hAb-ETB-CSLAHNPs were stable in 50% serum for up to 12h.

The specific aim of Chapter 5 was to evaluate the cellular uptake, intracellular trafficking, and cellular viability of ETH-CSLAHNPs and targeted hAb-ETB-CSLAHNPs in two human lung adenocarcinoma cells, A549 and HCC827 cells. A549 cells express low levels of wild-type EGFR whereas HCC827 cells express higher levels of EGFR due to the acquired mutation in the EGFR tyrosine kinase domains. For intracellular imaging, 1 mol % of NBD-PC was incorporated into CSLAHNPs. The

targeting ability of hAb-ETB-CSLAHNPs was confirmed by cell association study. Confocal microscopy and flow cytometry results showed higher uptake of hAb-ETB-CSLAHNPs and ETB-CSLAHNPs in HCC827 cells compared to A549 cells up to 4 h. Endocytosis inhibitor assay showed major mechanism of particle uptake was *via* caveolae-mediated endocytosis. Significantly higher reduction in cell viability was observed for both targeted and untargeted ETB-CSLAHNPs compared to ETB-solution form in HCC827 compared to A549 cells. Similar results were observed for colony formation assay. Western blot results showed significant downregulation of phosphorylated EGFR after ETB-CSLAHNPs treatment compared to control. The results suggest the potential therapeutic advantages of targeted and untargeted ETB-CSLAHNPs over ETB in solution in EGFR-overexpressing NSCLC.

The final aim of the project was to prepare lyophilized CSLAHNPs and also to perform the storage stability for both liquid dispersion and lyophilized solid powder. The present study also showed that CSLAHNPs can be stabilized both during the lyophilization process as well as for long-term storage using adequate concentrations of suitable lyoprotectants. Among two disaccharides (sucrose and trehalose) used as lyoprotectants, sucrose was the best in preserving the cake properties and physicochemical characteristics at 1:16 weight ratio of CSLAHNPs to sucrose. Accelerated temperature storage at 25°C had a negative influence on both lyophilized and liquid form stability. The study also showed that the lyophilized form retained the physicochemical properties CSLAHNPs compared to the liquid dispersion form at 2-8°C storage condition for 60 days of storage.

Overall, the results suggest successful design and development of targeted hAb-ETB-CSLAHNPs and untargeted ETB-CSLAHNPs. Various formulation and process variables were optimized to obtain prototype formulation with acceptable physicochemical properties including mean size, polydispersity index, zeta potential, drug loading efficiency and drug release profiles. The study also showed the potential therapeutic advantages of targeted and untargeted ETB-CSLAHNPs over ETB in solution in EGFR-expressing NSCLC cells. Lyophilized power of CSLAHNPs containing sucrose exhibited refrigerated storage stability for 2 months retaining acceptable physicochemical properties. Therefore, the overall experimental data supported the central hypothesis of improved therapeutic efficacy of anti-EGFR half-antibody conjugated erlotinib loaded CSLAHNPs as a potential targeted drug delivery system for the therapy of non-small cell lung cancer.

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