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Abstract

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A validated isocratic HPLC method was developed and validated for the quantitative determination of AMB. Design D was determined to be the lead formulation with drug loading of 13.6 ± 6.9 of AMB/mg of IONP. The size, ζ -potential, and PDI for all formulation designs were found to be in an optimum range for a nanomedicine with ≤ 36 nm, ~ -20 mV, and ≤ 0.2 , respectively. The TEM images confirmed that the nanoparticles were monodispersed and spherical in shape. The drug release profile indicated a burst release up to 3 hours for designs A and B, followed by a sustained drug release profile up to 72 hours. Designs C and D (with and without glutaraldehyde) also had a sustained drug release profile up to 72 hours. The major mechanisms of drug release from these formulations were determined to be Fickian and non-Fickian diffusion with first order and Higuchi kinetic models as best fit.

The cellular uptake profile for design D exhibited a time dependent uptake with maximum uptake at 0.5 and 4 hours for *C. albicans* and *C. glabrata*, respectively. All designs exhibited improved efficacy over AMB-D in the susceptibility testing conducted on clinical isolates of *Candida*. Design D was found to have an enhanced killing ability and was 16-25 fold more efficacious than AMB-D. An in vitro cellular association study found the uptake mechanism was energy dependent. An endocytosis inhibitor evaluation determined the major particle uptake pathway for *C. albicans* was lipid-raft mediated endocytosis, whereas for *C. glabrata*, it was clathrin-, caveolar-, and lipid-raft-mediated endocytosis.

TEM and confocal images provided evidence the AMB-IONP were localized at or near the cell wall and membrane wall and inside the cytoplasm, nucleus and endolysosomal vesicles for tested isolates. The lyophilized formulation of AMB-IONP was successfully prepared using an appropriate amount (1:16 to the weight IONP) of the of lyoprotectant, sucrose. A short term stability study of both formulations (lyophilized and aqueous dispersion) at 5°C and 25°C for up to two months showed the lyophilized form was stable.

In conclusion, a targeted nanotheronostic drug delivery system (AMB-IONP) was successfully designed, developed, characterized and evaluated as a potential drug product for IFIs treatment.

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**Development and Evaluation of Amphotericin B Loaded Iron Oxide Nanoparticles
for Targeted Drug Delivery to Systemic Fungal Infections**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

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

By
Pavan Balabathula
December 2015

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DEDICATION

I dedicate this dissertation to my family. There is a special feeling of gratitude to my affectionate parents, Chakrapani and Prameela Balabathula whose continuous support and encouragement was remarkable. More than anyone else, it was my father's ambition and determination that encouraged me to pursue a doctoral degree. His passion towards education will always inspire me to achieve greater things. My mother always has goals in her prayers and gave me extra strength to achieve my ambitions. I could not have achieved success and honor without both of you.

I also dedicate this dissertation to my amazing wife, Jaya and wonderful daughter, Aadhya. My wife's sacrificial care for me and my daughter made it possible for me to complete this work. Jaya constantly motivated and helped me to keep focused on my goals. My little daughter always rejuvenated my energy, giving me the required strength to perform. Both of you have been my best cheerleaders.

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LIST OF ABBREVIATIONS

ζ	Zeta
AIDS	Acquired immune deficiency syndrome
ALL	Acute lymphoblastic leukemia
AMB	Amphotericin B
AMB-D	Amphotericin B deoxycholate
AMB-IONP	Amphotericin B loaded iron oxide nanoparticles
AUC	Area under the curve
BIC	Bio-ionomer complexes
BSA	Bovine serum albumin
CA	Camptothecin
CDC	Centers of Disease Control
CLSI	Clinical and Laboratory Standards Institute
CNS	Central nervous system
CNT	Carbon nanotubes
CSF	Cerebrospinal fluid
CV	Coefficient of variation
DAPI	4', 6-diamidino-2-phenylindole
DLS	Dynamic light scattering
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine
DMSO	Dimethyl sulfoxide
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE	1,2-di-(9 Z- octadecenoyl)-sn-glycero-3-phosphoethanolamine
DOX	Doxorubicin
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine
DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
DSC	Differential scanning calorimetry
DSDMA	bis(2-methylacryloyloxy) disulfide
DSPC	1,2-distearoyl-sn-glycero-3-phosphatidylcholine
DSPE	1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine
DTT	Dithiothritol
EDAC	1-ethyl-3-(3- dimethylaminopropyl) carbodiimide
EPR	Enhanced permeation and retention
FBS	Fetal bovine serum
HCPT	10-hydroxycamptothecin
HPLC	High Performance Liquid Chromatography
HSA	Human serum albumin
HSPC	Hydrogenated soy phosphatidylcholine
IFIs	Invasive fungal infections

IONP	Iron oxide nanoparticles
IV	Intravenous
LOD	Limit of detection
LOQ	Limit of quantification
LPD	Lipid protamide DNA
LUV	Large unilamellar vesicles
MDR	Multi drug resistant
MePEG	Methoxypoly(ethyleneglycol)
MFI	Mean fluorescence intensity
MIC	Minimum inhibitory concentration
MLV	Multilamellar vesicles
MOPS	3-(N-morpholino)propanesulfonic acid, 4-morpholinepropanesulfonic acid
MPP	MPEG-PLA
MPS	Mononuclear phagocytic system
MRI	Magnetic resonance imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVV	Multivesicular vesicles
MWCNT	Multi walled carbon nanotube
M β CD	Methyl- β -cyclodextrin
NGR	Asn-Gly-Arg peptide
NHS	sulfo-N-hydroxysuccinimide
NIH	National Institutes of Health
NIPAM	N-isopropylamine
NIR	Near infra red
NP	Nanoparticle
PAEMA	poly(2-aminomethacrylamide)
PAMAM	polyamidoamine
PBS	Phosphate buffer saline
PCL	poly(caprolactone)
PDI	Polydispersity index
PDLLA/ PLA/ PLLA	poly(D,L-lactic acid)
PE	phosphatidylethanolamine
PEG	poly(ethyleneglycol)
PEI	Polyethylene imine
PEO	poly(ethyleneoxide)
PMA	Poly(methacrylic acid)
POO	Polypropylene oxide
PPO	poy(propyleneoxide)
PTX	Paclitaxel
r ²	Coefficient of determination

RES	Reticuloendothelial system
RGD	Arginine- glycine-aspartic acid
RPMI	Roswell park memorial institute
SD	Standard deviation
siRNA	Small interfering RNA
SLN	Solid lipid nanoparticle
SSLN	Stealth solid lipid nanoparticle
SUV	Small unilamellar vesicles
SWCNT	Single walled carbon nanotube
TAE	Tris-acetate-EDTA
TEM	Transmission electron microscopy
VEGF	Vascular endothelial growth factor
5-FU	5-florouracil

CHAPTER 1. INTRODUCTION*

1.1. Statement of the Problem

1.1.1. Systemic fungal infections that have infected the CNS

Invasive mycoses are a significant health problem. Fungal infections are widespread throughout populations of humans and animals. Once infected and spread to the central nervous system (CNS) the infection is often difficult to cure [1]. Complications can include raised cerebrospinal fluid (CSF) pressure and growths with associated edema resulting in high mortality rates. For example, global burden of cryptococcal meningitis has been projected to kill 625,000 patients annually out of one million cases [2]. In sub-Saharan Africa, it is estimated there are over 500,000 deaths every year. Recently, a 2012 meningitis outbreak caused by contaminated fungal methylprednisolone injections from the New England Compounding Center [3, 4] and distributed to 76 locations in 23 different states in the USA resulted in 751 patients diagnosed with fungal meningitis and 64 deaths.

Systemic or invasive fungal infections (IFIs) are particularly a problem in immunocompromised HIV patients [5]. IFIs such as CNS fungal infections were once regarded as rare infectious diseases but with the greater use of corticosteroids and cytotoxic drugs, the AIDS epidemic has increased the frequency of CNS mycoses [6]. Patients already have a potentially life threatening disease, AIDS, but may also be coinfecting with a fungal disease or tuberculosis. HIV associated cryptococcal meningitis is rapidly fatal often with no patients surviving longer than 30 days after diagnosis without aggressive treatment [1]. Therapy is further complicated with antiretroviral therapy for AIDS with the possibility of immune system rebound. Antifungal therapy for immunocompromised patients is usually divided into induction, consolidation and maintenance phases. During induction the primary goal of antifungal pharmacotherapy is to achieve rapid fungicidal activity and CSF sterility. To achieve fungal eradication rapidly, the drug must enter the CNS tissue rapidly, and be efficient at killing the pathogen within 14 days.

A number of fungi may cause infection in normal humans such as *Candida* species, *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Coccidioides immitis* [7]. Most fungi are opportunistic and can infect immunocompromised hosts. *Candida albicans* is in the normal flora of the human mucus but is known to access the brain through nasal passages. However, most other fungi enter the body through breathing or

* Adapted with permission. Bhattacharjee, H., P. Balabathula, and G.C. Wood, *Targeted nanoparticulate drug-delivery systems for treatment of solid tumors: a review*. Ther Deliv, 2010. 1(5): p. 713-34.

skin scrapes. Eventually, macrophages can take up the fungus and sometime carry it across barriers into the CNS. Various species cause different abnormalities. Acute or neutrophilic meningitis has been seen in *Candida* meningitis. *Cryptococcus neoformans* causes a lymphocytic meningitis and *Coccidioides immitis* causes a granulomatous meningitis [7].

1.1.2. Antifungal therapy

IFIs are causing increased morbidity and mortality for immunosuppressed patients with up to 62% mortality rates [2]. They have been associated with diverse pathogens and the unavailability of sensitive diagnostic protocols, leads to a significant challenge [8]. Therefore, more often the treatment options are experiential at least until the causative pathogen is identified along with susceptibility profiles.

In response to these life threatening fungal infectious diseases there are several antifungal agents that have emerged over the last six decades. Unfortunately, there is no ideal antifungal drug available for therapy. Newer drug products are becoming available and some are less toxic as compared to older agents. These drug products include the newer azoles and echinocandins. However some of these are associated with problems of their pharmacokinetic profiles, spectrum of activity, route of administration, and drug interactions, thus limiting their applicability [8].

The first wide spectrum antifungal agent developed was amphotericin B-deoxycholate (AMB-D) in 1958 by Squibb Laboratories, it is still considered a gold standard for antifungal therapy for many years in spite of its association with high incidences of infusion-related adverse effects and dose-limiting renal toxicity [8-11]. Infusion-related adverse events include nausea, vomiting, fever, thrombophlebitis, chills or rigors, anaphylaxis, and dyspnea. Amphotericin B (AMB) causes arteriolar vasoconstriction that causes renal ischemia and decreased glomerular filtration rate [12]. These adverse events make AMB-D unfavorable for treating IFIs. However, it still remains the drug with broadest spectrum of activity among available antifungal agents. In an attempt to minimize these toxic effects lipid formulations (AmBisome®, Amphocil®, and Abelcet®) [6] of AMB were developed, but the relative safety and efficacy of these formulations still remains questionable [8]. Additionally the lipid formulations are associated with increased cost [13]. For instance the estimated daily cost for an average patient is \$10-17 for Fungizone®, whereas for Ambisome® it ranges from \$790-1316. This lead to further investigations for equally potent with minimum toxicity drug products that could be given both orally and intravenously. The timeline for evolution of different antifungal agents are shown in **Figure 1-1** [9]. However these agents suffer from several disadvantages such as lack of broader spectrum activity (ketoconazole, fluconazole), varied pharmacokinetic profile (posaconazole and voriconazole), erratic absorption or toxic gastrointestinal effects (itraconazole), risk of drug interactions (voriconazole and posaconazole), and lack of potency towards some common opportunistic pathogens including species of *Aspergillus*, *Fusarium*, and *Cryptococcus* [9]. AMB products are not known to cause antifungal resistance [14]. In contrast, azole

Figure 1-1. Timeline of systemic antifungal agents

antifungal treatment for some resistant species of *Candida* appears to becoming a more consistent problem. In spite of significant amount of evolution towards the improved systemic antifungal therapy over the last sixty years, the current antifungal therapy cannot live up to all the expectations. Currently there is no single antifungal drug, which is considered optimum for all patients suffering with a specific fungal infection due to the comorbidity in each patient, risk of drug interactions, infection site problems, and immunodeficiency disease [9].

AMB belongs to class of polyene antibiotics that was first extracted from South American soil *Streptomyces nodosus* (gram positive bacteria) through a fermentation process [8, 13, 15]. It has broad spectrum of activity against fungal pathogens and is considered as the first line drug for life threatening systemic fungal infections including pathogens *Candida albicans* and *Aspergillus fumigatus*. AMB is a yellow to orange colored compound, which exhibits amphipilic and amphoteric characteristics. It contains a polyene chain at one end and a relatively polar groups, which are asymmetrically organized making it as amphipilic (**Figure 1-2**). It has two ionizable polar groups, COOH and NH₂ to exhibit AMB as amphoteric. Due to these properties AMB has poor solubility in both aqueous and most organic solvents. When pH is considered, it is poorly soluble around the physiological pH of 7.4. AMB does show good solubility at extreme pH conditions at less than 2 and more than 11, but at these conditions AMB is unstable. This solubility profile make it hard to formulate for better efficacy with reduced toxicity. I would not want to formulate an injectable product at pH less than 2 or more than 11 under no circumstances.

1.1.3. AMB mechanism of action

There are several reports published in the literature regarding possible models for the mechanism of action of AMB [16]. AMB exerts its fungicidal activity by compromising the integrity of fungal lipid membranes. These yeast membranes are composed of ergosterol, which are structurally close to mammalian sterol, cholesterol. Ergosterol is essential for lipid bilayer stability and for dynamic properties. Though mammalian cell membranes contain cholesterol, AMB has more strong binding affinity towards the yeast fungal membrane sterol, ergosterol, which accounts for its targeting and killing effectiveness when treating deep fungal infections in immunocompromised or transplant patients.

Chemically, AMB contains a macrolactone ring that is β -glycosylated with a mycosamine group as shown in **Figure 1-2** [16]. Structurally the ring is flat with seven conjugated double bonds on one side, which accounts for its chromophore character of yellow to orange color. The other side of the ring is composed of polyol subunit, the combination of these polar and non-polar moieties accounts for AMB's amphipathic character. The amphoteric moiety of the molecule comes from the amine group of the mycosamine and carboxyl group.

The first and widely reported mechanism of action of AMB is the ion-channel

Figure 1-2. Schematic representation of ergosterol, cholesterol, and AMB

pore formation model [17, 18]. This model is depicted in **Figure 1-3a**, which explains that AMB molecules simply binds ergosterol and organize in such a way that they form a barrel in the lipid bilayer with polyene moiety pointing outwards and polyol moiety facing inward. These barrels can be either single (half pore) or double barrel formations, but either way this form a transmembrane channel leading to leakage of the K^+ and other intracellular components that are essential for cell function. Eventually this process leads to fungal cell death. In the second model AMB causes cell damage due to oxidation, which leads to lysis of red cells [19]. Eventually this will cause the formation of reactive oxygen species such as hydrogen peroxide, hydroxyl radicals, and superoxide, thus oxidizing the lipid membrane, thereby affecting the function of the cell. AMB can also cause cell death through an intracellular oxidative burst [20]. The third model demonstrates that AMB get adsorbed on the surface of the lipid membrane oriented parallel to the lipid plane (**Figure 1-3b**). This will trigger to sequestration of the ergosterol in the membrane surface leading to membrane destabilization and finally cell death [16]. The last and recently reported mechanism of action is based on sterol sponge model, where AMB forms a large extra membranous aggregates [21]. These aggregates tend to extract the ergosterol from lipid bilayers to produce a fungicidal action (**Figure 1-3c**). This mechanism requires strong interaction among AMB and ergosterol.

The mostly widely accepted AMB mechanism of action is related to both ergosterol binding and pore formation [16]. But some reports indicated that the key factor for fungicidal action is related to sterol binding of AMB and pore formation is one of several mechanisms depending on AMB sterol binding [18, 22]. The concentration of AMB in the lipid bilayer plays an important role in channel/pore formation and whereas the sterol has less influence. Even the pore size is dependent on the AMB concentration in bilayer. Thus, more drug at the site of infection or in lipid bilayer means more killing effectiveness. Hopefully this can be achieved with more focused delivery through targeted nanoparticulate drug delivery system. Since, AMB has strong binding affinity towards the ergosterol and this is required for fungicidal action, AMB itself can be potentially used as both a targeting ligand and effective antifungal.

1.1.4. Ideal antifungal therapy

With the high rate of morbidity and mortality seen with IFIs such as CNS fungal infections treatment, selection can be critical. The treatment is influenced by multiple factors such as the condition of the host, the pathogen and the drug properties [7]. Key among these is the drug's abilities to enter and act in the brain and spinal cord. AMB is a polyene antibiotic that binds to fungal ergosterol causing leakage and cell death. AMB and flucytosine in combination are used to treat *Candida* and *Cryptococcus neoformans* CNS infections. Unfortunately, AMB has significant hepatic, renal, and cutaneous side effects [9]. Infusion reactions and bone marrow suppression producing an anemia with decreased epoetin production have also been reported. If just some of the adverse effects of AMB could be ameliorated with a new delivery system, this would be considered a significant therapeutic advantage.

Figure 1-3. Schematic representation of different proposed mechanism of actions of AMB

Reprinted with permission from Springer. Kamiński, D.M., *Recent progress in the study of the interactions of amphotericin B with cholesterol and ergosterol in lipid environments*. European biophysics journal, 2014. **43**(10-11): p. 453-467. **(a)** represents ion channel model where AMB molecules aggregate to form a barrel/pore with its polyhydroxy chain groups pointing inward and its polyene moiety pointing outward. **(b)** represents surface adsorption model where AMB extracts ergosterol from the lipid bilayer to the surface of the membrane there by compromising the integrity of the cell membrane. **(c)** represents sterol sponge model where AMB forms large aggregates to extract ergosterol from the lipid membrane.

Over the last 15 years our laboratories and other labs have developed unique designs for nanoparticle drug products. The evidence showed that targeted colloidal (nanoparticle) drugs (i.e., nanomedicines) can impart significant therapeutic advantages over traditional true solution IV drug delivery systems [23-25]. For example, highly toxic drugs (e.g., selected anticancer agents and antibiotics) in nanoparticle encapsulated delivery systems, can be made less toxic by targeting and protecting the particles until they reach the affected tissues since the body is not flooded with toxic true solution drugs that distribute to healthy tissues causing adverse effects. Previous animal studies suggested the nanomedicine total dose can be cut 20-30% while maintaining effectiveness (when compared to standard IV solution therapies) [26]. Examples include liposomal doxorubicin injection that helps to protect the heart from cardiotoxicity [27]. Encapsulated drug usually means less systemic toxicity [28] and encapsulating materials are biodegradable and nontoxic. Targeted nanoparticles can produce an extended release effect, often lasting 1.5-3 days in the body rather than a few hours (as with IV true solution drugs). Targeted nanoparticle drugs can be programmed to deliver drugs to cell surface, cytosol, organelle or nucleus receptors. A significant therapeutic advantage is that nanoparticle drugs are not affected as much as true solution drugs or as fast by exotransporters such as Pgp allowing more drug longer at/in the affected cells [29-31]. Overcoming this type of drug resistance is a significant problem in cancer chemotherapy and may also be a problem with certain anti-infectives.

Nanoparticle drug delivery systems can provide multiple functions simultaneously, (i) Multiple drugs or a drug and promoter can be incorporated into one delivery system. (ii) A drug and a diagnostic agent can be built into one nanoparticle delivery system (e.g., theranostic agents) [32, 33]. Design flexibility allows small molecule, protein or gene drugs to be delivered by targeted nanoparticle systems. Products can be lyophilized to create a reasonable product shelf life for clinical trials and marketplace use [34]. Batch size scale-up and manufacturing issues in sterile environments are becoming easier (and potentially cheaper) to overcome for certain types of nanoparticles, such as layered nanoparticles.

The decreased toxicity strategy is summarized in the concept that many adverse effects are related to either the total drug dose per day or per treatment cycle/s. If an antifungal can be made more effective per mg of drug, then the dosing regimen can be lowered thereby decreasing the incidence or severity of the adverse effect/s. Potentially this could mean that the dose of the improved product could be increased if drug resistance emerges or penetration of the drug is poor to the site of infection.

The issue of drug penetration to its site of action is critical for any drug to work. During distribution, drugs in true solution that are administered intravenously often have to bridge cellular barriers to reach the site of action. The ability of the barriers to pass or prevent entrance is related to the concentration, physicochemical properties of the drug, the barrier thickness and cell properties, and the existence of passive or active diffusion at the absorption/distribution site.

At infectious sites there is often active inflammation that may increase the

vascular permeability. Since the studies enclosed in this dissertation are related to small particles containing AMB as both a therapeutic and targeting agent. This design allows for higher concentration of the drug to be delivered to the infection site. The advantage of very small nanomedicine particles are that the particles deliver the drug in higher concentration at the infection sites without spreading the antifungal to many other tissues in the body creating more potentially deleterious effects.

Furthermore, AMB nanoparticles with iron oxide cores have a delayed and extended release at the infection site. The particles can be tracked by magnetic resonance imaging (MRI) imaging assuring the drug is reaching the sites of infection. Thereby, the location and residence time for the drug release can be tracked *in vivo* and the dosage regimen adjusted to the severity of the disease. Additionally, the ability to visualize the nanomedicine particles over time gives some idea of the time needed for the body to clear the drug in individual patients. Because of the design properties, the particles are expected to reach the infection sites with most of the drug intact where it is expected to slowly release from the albumin coating. If the release needs to be sustained the nanoparticle coating containing albumin can be cross linked to release the drug more slowly and prevent burst effects upon injection. Probably the largest advantage is that very small nanomedicine nanoparticles (about 30-40 nm) have a large surface area to release drug, are small enough to penetrate into potential CNS infectious sites and are in intimate vicinity or contact with the pathogen. Nanomedicines in this particle size range, have an advantage in that exotransporters such as Pgp actively facilitate molecular transport but often do not facilitate larger particle transport (nanomedicines) from infectious sites [29-31].

Some concerns related to infectious disease drug product design include:

- The new drug product is more effective than existing dosage forms. This is manifested in the product having therapeutic and/or economic advantages such as being more effective in eradicating a particular type of infection as compared to an existing or standard therapy.
- The new product has less or no deleterious adverse effects than existing products.
- The new product has pharmacokinetic advantages such as sustained release of the active ingredient leading to better patient compliance or less staff needs for product administration.
- The new dosage form should not be so complicated as to make scale up from laboratory batches to clinical trial size batches so technically difficult and/or expensive to prevent animal studies and/or clinical trials in humans.
- In cancer chemotherapy and infectious disease treatments, targeting of just the active pharmaceutical ingredient may not be enough. Therefore to increase therapeutic effectiveness may require the drug delivery system to also be targeted to the site of interest. This concept allows more drug to reach the site while keeping the total dose lower than the original product.

Our laboratory has been designing and testing nanomedicines for high mortality diseases including brain tumors, leukemias, lymphomas, lung cancers, acute lung injury

and infectious diseases. Most of the dosage forms incorporate active and/or passive targeting to increase effectiveness. Some of the products also have the ability to be tracked to the site of interest and therefore giving some assurance the drug product is at the site of interest and not spread throughout the entire body as most true solution parenteral drugs are after administration to the patient. For targeted delivery drug products the emphasis is not circulating plasma concentration of the drug but rather the concentration at the site of action. Decreasing the circulating concentration 20 to 30 percent to non-target organs/cells is often beneficial for toxic cancer and infectious disease drugs since this helps ameliorate potential adverse effects. Currently, there are no marketed targeted drug delivery systems available for IFIs and thus existing drugs cause significant dose related adverse reactions. For instance AMB-D causes renal toxicity and infusion related adverse reactions [11].

1.2. Nanomedicine

1.2.1. Nanoparticles in treatment of deadly diseases

Nanoparticles are commonly described as colloidal particles with a size range of 10 to 100 nm [24]. They are macromolecular constructs generally consisting of polymeric moieties in which a drug or a biological active material is dissolved, entrapped, encapsulated, or even attached or adsorbed to the external surface [35]. The systems being evaluated or used in current targeted cancer therapy options are prepared employing various biodegradable or non-biodegradable materials such as polymers, metals and ceramics. Based on the raw materials used in their preparation and their manufacturing method, these nanoparticles can be formed in different shapes and sizes, and with varying surface characteristics. Morphologically, nanoparticles can be spherical, branched or shell structures with unique characteristic properties for drug delivery. To overcome the limitations of traditional chemotherapy and gene delivery, numerous nanoparticulate drug delivery systems have been designed with unique physical and biological properties [36]. To date, many types of nanoparticles have been characterized such as quantum dots, dendrimers, micelles, liposomes, carbon fullerenes and polymeric nanoparticles.

1.2.2. Mode of action

Traditional chemotherapeutic agents used for the treatment of solid malignancies are generally considered to be effective in suppressing tumor growth but are associated with significant adverse effects. The highly toxic drugs used in these therapies have an inherent shortcoming because such therapies are unable to distinguish between the cancer cells and normal cells. Therefore, a therapy that could effectively eliminate tumor cells without damaging the surrounding healthy tissues would be extremely beneficial.

Nanoparticles as drug delivery systems have a number of advantages over

traditional chemotherapeutic agents. The ability of nanoparticles to encapsulate highly toxic drugs at high concentrations is advantageous as it increases its payload and also reduces the exposure of the drug to the normal tissue. Drugs that are generally incorporated into such systems also tend to escape the metabolic pathways that can degrade drugs and render them inactive. Thus, encapsulation offers an opportunity for reducing the dose required for therapeutic effect, especially if these particles are targeted. Nanoparticles can also be surface-modified to escape the reticuloendothelial system (RES) [37] and serve as a surface for attachment of targeting ligands thereby initiating a site- or cell-specific response increasing the effectiveness of the treatment regimen.

A number of nanoparticle drug delivery systems have been evaluated for cancer therapeutics [38]. These systems are based on the assumption that the drug being carried in the nanoparticle system can offer a high drug concentration at the tumor site due to its specificity and also have a low systemic exposure. The majority of these particles have extensive surface modifications with the main characteristic of having a hydrophilic exterior surface in order to impart stealth properties and enhance circulation [39]. Moreover the charge characteristics of these particles, usually positive charges, can also aid in the process of endocytosis [40].

Such surface modifications prevent the adherence of nanoparticles to serum proteins and aid in increasing the circulatory time. The process of opsonization, binding of nanoparticles to serum proteins, is a major factor influencing biological activity and has a crucial role in the efficiency of nanoparticulate drug delivery systems. Conventional nanoparticles, when administered via the intravenous route, rapidly bind to opsonin proteins present in the blood serum. This binding process allows macrophages of the mononuclear phagocytic system (MPS) [41] to easily recognize and eliminate nanoparticles even before they can reach their target site. In order to circumvent this process, several methods have been developed to mask nanoparticles from the MPS. One option to prevent this process is the adsorption or grafting of poly(ethyleneglycol) (PEG) to the surface of nanoparticles. Surface modifications using PEG or PEG-containing copolymers result in an increase in the blood circulation half-life of the particles by several orders of magnitude. This modification creates a hydrophilic layer around the nanoparticles capable of preventing opsonin proteins from binding to nanoparticles through steric repulsion forces [42].

Two types of targeting theories have been widely accepted for nanoparticles. These include passive targeting and active targeting.

1.2.3. Passive targeting

Normal capillaries consist of endothelial cells, which are dynamic in nature. Such normal tissues manifest a 'basal' microvascular permeability that is defined as the permeability of microvessels to fluid and hydrophilic solutes. The basal permeability is a constant process that occurs with small physiological variations by mechanisms that are largely unknown [43].

Most malignant solid tumors have the ability to grow at faster rates than the normal surrounding tissues. In order to support this high rate of growth, the blood circulation around the tumor is increased to carry nutrients to the solid tumor. In such tissues, increased microvascular permeability occurs and correlates to a large increase in permeability of fluid and plasma proteins. It is not known whether the increased permeability is an exaggeration in rate of the basal mechanisms or if it occurs via completely different mechanisms [43].

The tumor initiates the process of forming new blood vessels (angiogenesis) by enhancing expression of growth factors such as vascular endothelial growth factor (VEGF) [44]. Studies of tumor angiogenesis have indicated that tumor cell aggregates in the size range of 150–200 μm , begin to show dependence on the neovasculature for its blood supply [45]. The rapid rate of angiogenesis in the tumor tissue leads to abnormal formation of the vasculature resulting in poorly aligned defective endothelial cells with wide fenestrations [46], lack of a smooth muscle layer in some tumors [47, 48], or innervations [48], and impaired vasomotor functions. Additionally, the tumor tissues usually lack effective lymphatic drainage. All these factors lead to an abnormal microvasculature that is leaky and is accompanied by aberrant molecular and fluid transport dynamics.

The altered fluid dynamics are accentuated when nanoparticulate drug delivery systems and certain macromolecular drug molecules flow through these leaky vasculatures. Targeting of drugs using nanoparticulate drug delivery systems usually consists of particles that are less than 100 nm in size (usually approximately 80 nm). Matsumura and co-workers have conducted studies that show that polymeric nanoparticles are more permeable and transport efficiently across the endothelial barrier in the tumor vasculature and are retained because of the poor lymphatic drainage [49]. This phenomenon was later named as the ‘enhanced permeation and retention’ (EPR) effect (**Figure 1-4**).

Targeting using EPR is significantly dependent on the size and the surface characteristics of the nanoparticle. It is believed that, if the nanoparticle drug delivery systems are allowed to circulate in the bloodstream for a sufficient amount of time, a larger fraction of these particles will eventually reach the tumor. The EPR effect should not be considered as an immediate effect; the poor lymphatic drainage also seems to potentiate the effect and directly relates to prolonged drug retention for an extended period of time [50].

1.2.4. Active targeting

Every cell in the human body carries distinct cell surface markers, and this is true for tumor cell too. Interestingly, certain cell surfaces [51] or tissue markers are differentially expressed in only tumor cells and are absent or exist in very low levels of expression in the normal healthy tissue [52]. Thus the tumor cells can be selectively targeted by using ligands that have a high binding affinity for these cell surface or tissue

Figure 1-4. Enhanced permeation and retention effects

(A) Healthy tissue with normal vasculature, smooth muscle development and no evidence of fenestrations. Nanoparticles generally do not pass through the endothelial membrane to extravagate into the healthy tissue. **(B)** Malignant tissue with abnormal vasculature, smooth muscles and wide fenestrations. Nanoparticles in the acceptable size range extravagate in to the tissue and are retained for extended durations due to poor lymphatic drainage.

markers. Additionally, the ability to modify surface characteristics of nanoparticles allow the attachment of targeting ligands that can preferentially bind to the tumor cells at the target site [53].

Targeted nanoparticles have distinct advantages over their nontargeted counterparts. Nontargeted nanoparticles depend solely on their size, charge characteristics, leaky tumor vasculature and poor lymphatic drainage (EPR) for drug delivery. Although this type of drug delivery is superior to the traditional chemotherapeutic approach of administering stabilized solutions of drugs, it also has its share of disadvantages. The poor structural feature of the neovasculature on the tumor does not penetrate deep into the tumor mass, and any drug delivery systems will lack the ability to eliminate tumor cells present at the core of the tumor [54]. This dysfunctional blood supply to the tumor, coupled to the high interstitial pressure due to low lymphatic drainage, can cause the fluid in the interstitial spaces of the tumor to be forced out of the tumor mass and work against the diffusion of drugs from the blood vessel to the tumors [55, 56].

Modification of nanoparticles with targeting agents overcomes some of these shortcomings. A number of active tumor targeting approaches have been explored:

- Drug-loaded targeted nanoparticles that prevent the development of the tumor vasculature and starve the tumor by eliminating its source of nutrition;
- Targeted nanoparticles that bind to biomarkers [57] that are exclusively expressed on the tumor cell surface that may or may not be internalized by the tumor cells;
- Nanoparticles targeting common biomarkers that are present on both the tumor cells and the endothelial cells of the tumor vasculature.

Encapsulating antiangiogenic drugs in nanoparticles with surface ligands that target biomarkers on the endothelial lining of the blood vessels of the tumor vasculature, has been explored. The targeting ligands used in such approaches range from small homing peptides that contain Arginine- glycine-aspartic acid peptide (RGD) or Asn-Gly-Arg peptide (NGR) motifs [58] or monoclonal antibodies specific for endothelial markers [59]. RGD has been validated as a tumor homing peptide which selectively bind to integrin-binding peptides present on the endothelial lining of the tumor vasculature [58]. In a related study, Nallamothu and coworkers, designed and characterized long circulating liposomes containing combretastatin A4 (an anti-angiogenic agent) surface modified with RDG [60]. The results from another related study indicated that targeted liposomes showed significantly higher binding to their target cells than non-targeted liposomes, presumably through specific interaction of the RGD with its receptors on the cell surface [61].

In the second approach, targeting ligands specific to the tumor cells markers are tethered to the nanoparticles that also carry the drug payload. These targeting ligands include peptides, proteins, small molecules and antibodies. As the nanoparticles bind to these cell surface markers, they are generally taken up by the cells; once inside the cell the nanoparticles disintegrate and release the drug payload. Researchers have employed

RGD motif containing peptides that show specificity to certain types of cancer cells; for example, Zhao and co-workers evaluated RGD-based strategies for improving anti-tumor activity of paclitaxel-loaded liposomes in nude mice xenografted with human ovarian cancer, with encouraging results [62].

Exemplifying the third approach, another peptide, F3, has been used to target biomarkers that are commonly present in both the tumor vasculature endothelium and the tumor cells [63]. The F3 ligand has been shown to bind to nucleolin, which is present as an intracellular protein but is preferentially expressed by endothelial and tumor cells *in vivo*. The cell surface nucleolin is considered to be an important factor in angiogenesis and is a suitable target for drug delivery [64-66].

1.2.5. Types of nanoparticles

In this section we have attempted to classify and highlight important aspects of the different nanoparticulate systems available either as approved drug delivery systems or products being evaluated as potential drug delivery systems. By employing these delivery systems, we can achieve superior efficacy of currently used cancer drugs and advance the field of targeted site specific controlled drug delivery. Developments in nanoparticulate drug delivery systems for the treatment of solid malignancies are provided in **Table 1-1**.

1.2.5.1. Micelles. Micelles are supramolecular nanoparticulate structures that consist of a core and a surrounding shell. They have significant advantages for cancer diagnosis and therapy. These systems have been explored extensively for their relatively small size (10–100 nm), ability to solubilize both hydrophobic drugs and imaging agents, and prolonged pharmacokinetics. They provide a useful starting point for development of nanoparticle chemotherapy agents (**Figure 1-5**) [67]. Micelles are structures formed when certain polymeric molecules or ions, such as amphiphiles or surfactants, are introduced in a liquid medium at an appropriate concentration. The ability of these molecules to have two distinct regions in their molecular structure that constitute a hydrophobic tail and a hydrophilic head, results in the molecules having opposing affinities. At low concentrations in a liquid medium they tend to exist as monomers and have a sub-colloidal distribution. As their concentration increases, they form aggregates that have a monolayer of 50 or more monomers and are known as micelles. Micelles are spherical with a hydrophobic core and a hydrophilic exterior shell or corona in aqueous solution (**Figure 1-6**) [67]. Although this is the most common structural configuration, some researchers have also evaluated reverse micelles; the hydrophobic regions form the exterior whereas the hydrophilic head group forms the core of the micelles (**Figure 1-6**) [68].

Micelles have been extensively explored as nanoparticle drug carrier systems. Traditionally, micelles have been used in drug product development as a method of solubilization for hydrophobic drugs. In today's cancer therapy research environment,

Table 1-1. List of selected nanoparticles evaluated/explored as nontargeted or targeted drug-delivery systems for the treatment of solid malignancies

Type of NP	Size range	Application	Drugs explored	Selected references
Micellar	10-100 nm	Drug delivery and solubilization	Paclitaxel, Doxorubicin Docetaxel, oligonucleotides	Blaco et.al. [67, 69] Kim et. al. [69], Mu et.al. [70]
Liposomal	50-400 nm	Drug delivery and diagnostic aid	Paclitaxel, Combretastatin A4, Doxorubicin, oligonucleotides	Ogawara et. al. [71], Nallamotheu et. al. [61], Hu et. al. [72], Bruckheimer et. al. [73],
Nanoemulsions	20-600 nm	Drug delivery	Dacarbazine, Camptothecin, Doxorubicin, Tamoxifen, Aclacinomycin	Tagne et. al. [74, 75], Bouchemal et. al. [76], Ohguchi et. al. [77] , Han et. al. [78]
Nanocells	120-200 nm	Drug delivery	Combretastatin, Doxorubicin	Sengupta et. al. [79]
Solid lipid NP	100-400 nm	Drug delivery and contrast agents	Doxorubicin, Camptothecin, 5-FU, Paclitaxel, oligonucleotides,	Yu et. al. [80], Chen et. al. [81] , Zara et. al. [82], Oyewumi et. al. [83]
Carbon Fullerenes		Drug Delivery and contrast agents	Doxorubicin, Paclitaxel	Welsher et. al. [84], Liu et. al.[85] , Wu et. al. [86], Zhang et. al. [87]
Dendrimers	1 -10 nm	Drug delivery and contrast agents	Doxorubicin, Methotrexate, si RNA, Antibodies, Paclitaxel	Majoros et. al. [88], Ooya et. al. [89], Bhadra et. al. [90], Zhu et. al. [91], Lim et. al. [92], Wiener et. al. [93]

NP: Nanoparticle.

Figure 1-5. Active targeting concepts

Ligands that are specific to the biomarkers expressed on cell surfaces of the tumor vasculature or on the tumor cells can be employed to target nanoparticles. Such an approach would be termed as active targeting. The ligand on the NP surface can be considered as the ‘hitch’ while the NP would be the ‘trailer’ carrying the drug payload. This figure explains the three approaches explored to achieve active targeting. **(A)** NPs with specific ligands on the exterior surface that have affinity towards endothelial cell surface markers; **(B)** targeted NPs transmigrating through wide fenestrations in the endothelial lining; **(C)** NPs with specific ligands on the exterior surface that have affinity towards tumor cell surface markers. NP: Nanoparticle.

Figure 1-6. Micelles

(A) Indicates the structural feature of a micellar unit, amphiphile, which is characterized by a nonpolar/hydrophobic tail and a polar/ hydrophilic head; (B) shows a cross-sectional view of a micelle; (C) structure of a reverse micelle: the hydrophilic head forms the core whereas the hydrophobic tail forms the exterior of the micelle; (D) structure of a regular micelle: the hydrophobic tail forms the core whereas the polar head forms the exterior of the micelles.

micelles that are studied employ precisely engineered amphiphilic polymers (e.g., block polymers). In the presence of an aqueous environment, these polymers form supramolecular biocompatible structures that have the ability to incorporate drugs into their core [94]. Micellar systems using such modified block polymers have been shown to have several advantages over some other nanoparticle drug delivery systems for treatment of solid tumors. These advantages include controlled drug release modulated by the polymers or amphiphiles used, reduced uptake by the RES systems, and enhanced EPR effects [70].

The nature of the micelle dictates its viability as a chemotherapeutic drug delivery system that in turn is dependent on the polymers used to form the micelles. Researchers have designed, developed and evaluated a number of polymers that are biocompatible and biodegradable. The most commonly used outer shell or corona-forming polymer is PEG with a molecular weight in the range of 2 to 15 kD [67]. Core-forming polymers used to form micelles include poly(D,L-lactic acid) (PDLLA), poly(ϵ -caprolactone) (PCL), poly(L-aspartic acid) and polypropylene oxide (POO). Studies exploring the nature of certain block copolymers such as poly(ethylene oxide)-*b*-poly(ϵ -caprolactone) (PEO-PCL) [95], methoxy poly(ethylene glycol) (MePEG) [96], poly(ethylene oxide)-*b*-poly(methacrylic acid) (PEO-PMA) [69], poly(ethylene glycol)-*b*-poly(lactic acid) (PEG-PLA) [97], and poly(ethylene glycol)-*b*-poly(ϵ -caprolactone) (PEG-PCL) [97] have yielded useful information with respect to their drug delivery potentials for the treatment of cancers. Many of the polymeric micelle systems can also be surface modified to carry targeting ligands for tumor specific targeting (**Figure 1-7**). Monoclonal antibodies [98], small peptides [99], and small molecular ligands [100] have been explored for active targeting of micelles.

The use of micellar nanoparticles as an option for cancer treatment is exemplified by a number of studies reported in the literature. Mu and co-workers, in a recent study, reported the design of a mixed micelle system that comprised of MPEG-PLA (MPP) and Pluronic® copolymers [70]. This system has been developed for enhancing the bioavailability and overcoming multidrug resistance of docetaxel in cancer therapy. *In vivo* pharmacokinetics study in rats employing this diblock-triblock copolymer system showed that the mixed micelles significantly enhanced the bioavailability of docetaxel compared to the commercial docetaxel dosage form Taxotere® (3.6-fold). Although most of the polymers used in micelle formation are biocompatible and biodegradable, control of drug release from these nanoparticles can often be a challenge. In order to control the rate and extent of drug release, Kim and co-workers have designed novel functional polymeric nanocarriers with ionic cores containing biodegradable crosslinks for delivery of chemotherapeutic agents [69].

In this study, block ionomer complexes (BIC) of PEO-PMA and divalent metal cations (Ca^{2+}) were formed and disulfide bonds were introduced into the ionic cores employing cystamine as a biodegradable crosslinker. The resulting crosslinked micelles with disulfide bonds represented soft, hydrogel-like nanospheres and demonstrated time-dependent degradation in a simulated intracellular reducing environment. The ionic character of the cores allowed a very high level of DOX loading (50% w/w) into the

Figure 1-7. Targeted micellar nanoparticle used in cancer chemotherapy or detection of solid tumors

Internal structure and the external surface modifications that can be engineered into the construct. The hydrophobic drug is encapsulated in the hydrophobic core that is made up of carbon chains. The external surface is surface modified with high-molecular-weight PEG to construct a hydrophilic corona that imparts stealth characteristics. This surface also aids in providing a platform to attach other targeting ligands for active targeting.

cross-linked micelles to be achieved.

In another attempt to control the release of drugs from the polymeric micelles, Oh and co-workers have developed unique flower-like polymeric micelles that can be programmed to deliver drugs at specific pH ranges. These pH-responsive systems comprise of structurally unique micelles that are approximately 160 nm in diameter and constructed from poly(N^{ϵ} -[3-diethylamino]propylisothiocyanato-L-lysine)-*b*-poly(ethyleneglycol)-*b*-poly(L-lactide) (poly[DEAP-Lys]-*b*-PEG-*b*-PLLA) [101]. The nanoparticles have been designed to have a self-assembled flower-like arrangement consisting of two hydrophobic blocks (deprotonated poly[DEAP-Lys] block and PLLA block) and a petal-like hydrophilic PEG block at physiological pH. As the pH decreases to slightly acidic pH (<pH 7.0), as in tumor extracellular pH, the flower-like micelles undergo a change in the hydrophobicity of the micellar core. The protonation of poly(DEAP- Lys) changes the physical property of the polymer from hydrophobic to hydrophilic, resulting in disintegration of the micellar core. The co-presence of a pH-insensitive PLLA block in the micellar core affects the protonation of poly (DEAP-Lys), allowing the micelle to be stable at pH 7.0–7.4.

Jiang and co-workers have developed micellar core cross-linked nanoparticulate systems that are responsive to thermal stimuli [102]. Upon exposure to elevated temperatures these particles degrade into bio-degradable and biocompatible monomers and effectively release the drug payload. In order to construct this system, well-defined poly (2-aminoethylmeth-acrylamide) (PAEMA) homopolymer was first synthesized in a controlled fashion. Core cross-linked micelles comprising of well-solvated PAEMA coronas and thermoresponsive cores were then obtained by copolymerization of *N*-isopropylacrylamide (PAEMA–NIPAM) and *bis* (2-methacryloyloxyethyl) disulfide (DSDMA) difunctional monomers. The study reported that in the presence of dithiothreitol (DTT), the obtained micelles could be broken down into monomers, due to the cleavage of disulfide cross-linkers; de-swelling of micellar cores could be achieved via heating above the phase transition temperature of PAEMA–NIPAM. The primary amine residues present on the corona of the micelles could be used as anchor points for specific targeting ligands for active targeting. A similar concept has been adopted by Skidan and co-workers where PEG–diacyl lipid micelles have been constructed loaded with the hydrophobic molecule meso- 5,10,15,20-tetraphenyl-21H,23H-porphine. This micellar system has been used for the photodynamic treatment of B-16 melanoma cells [103].

Liu and co-workers designed and constructed a series of amphiphilic oligonucleotides consisting of a hydrophilic oligonucleotide covalently linked to hydrophobic diacyl lipid tails [104]. These molecules in aqueous solutions spontaneously self-assemble into monodispersed, 3D micellar nanostructures with a lipid core and a DNA corona. Further evaluation of these systems showed that these types of micelles have excellent thermal stability and their size can be manipulated by altering the length of the DNA sequence. Moreover, in the micelle system, the molecular recognition properties of DNA are conserved and the DNA micelles can hybridize with complimentary sequences while retaining their structural integrity. Importantly, when interacting with

cell membranes, the highly charged DNA micelles are able to disintegrate themselves and insert into the cell membrane, completing the process of internalization by endocytosis.

1.2.5.2. Liposomes. Liposomes are artificially engineered nanostructures that exploit the natural arrangement of phospholipids to form small vesicles (**Figure 1-8**). The name liposome is derived from two Greek words: ‘lipos’ meaning fat and ‘soma’ meaning body. A liposome can be formed at a variety of sizes as unilamellar or multilamellar vesicles and usually consist of amphiphilic phospholipids. Structurally, liposomes can be envisioned as empty cells having a phospholipid bilayer. Such structures, similar to cells, have one layer of polar head groups constituting the exterior of the liposome with an affinity for aqueous environments. A similar layer of polar head groups arranges to form the interior of the liposomal structure entrapping an aqueous environment. The nonpolar hydrocarbon tails of the exterior layer face the hydrocarbon tails of the interior layer, forming a bilayer.

The biodegradable and biocompatible nature of liposomes has made them an attractive system for chemotherapeutic drug delivery. Anticancer drugs such as DOX (Doxil®/ Myocet®), AMB (AmBisome®) [105], and daunorubicin (Daunoxome®) [106] are currently being marketed in liposome delivery systems. Although clinically used liposomes range between 100 to 200 nm [107], liposomes can exhibit diameters ranging from 20 nm to 10 µm and exhibit surface charges. The variation in charge and size depend on their manufacturing process and type of phospholipids employed to construct the bilayer and type of liposomal system. The physicochemical characteristics of these nanoparticles, such as particle size, lamellarity, surface charge, sensitivity to pH changes and bilayer rigidity, can be manipulated. These particles can exist as small unilamellar vesicles (SUV) with particle size ranges between 20 nm and 100 nm, multilamellar vesicles (MLV) with particle size ranges between 100 nm and 5 µm, large unilamellar vesicles (LUV) with particle size ranges between 100 nm and 1 µm and multivesicular vesicles (MVV) with particle size ranges between 1 and 10 µm (**Figure 1-9**).

One of the significant advantages in using liposomes as a drug delivery system is that these particles undergo self-assembly under controlled conditions and in the presence of the right ingredients in a reproducible fashion [108, 109]. The encapsulation or incorporation of the drug in the liposomal vesicles is dependent on the type of phospholipids used, the processing temperature and the physicochemical characteristic of the drug (**Figure 1-8**). During the dynamic process of formation of the vesicles, encapsulation of the drug occurs.

Liposomes generally reach their site of action by extravasation into the interstitial space from the bloodstream dominated by the EPR effect. However, liposomal drug delivery to specific tissues occurs via active and passive targeting routes. Such dual targeting is achievable because these nanoparticles can be easily manipulated by adding additional molecules to the outer surface of the lipid bilayer. Surface modification of the outer surface of liposomes has mainly centered on the introduction of synthetic PEG phospholipids. The most commonly used phospholipids for the formation of liposomes

Figure 1-8. Liposome

Generally, hydrophobic drugs are incorporated in the hydrophobic bilayer of the liposomes consisting of hydrocarbon chains and the hydrophilic drugs are encapsulated in the aqueous core of the liposomal vesicle. The bilayer characteristics can be manipulated by addition of cholesterol to afford more rigid structures that are less leaky. Surface modification of these particles can render them invisible to the reticulo-endothelial system and mononuclear phagocytic system. High-molecular-weight PEG-1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine is employed to construct a hydrophilic corona that imparts stealth characteristics. The ability to surface modify these particles also help in the attachment of targeting ligands such as antibodies, cyclic peptides (arginine/glycine/aspartic acid) or folic acid on the outer surface.

Figure 1-9. Types of liposomes

(A) small unilamellar vesicles particle size ranges between 20 and 100 nm; (B) multilamellar vesicle with particle size ranging between 100 nm and 5 μm ; (C) large unilamellar vesicles with particle size ranging between 100 nm and 1 μm ; and (D) multivesicular vesicles with particle size ranging between 1 μm and 10 μm .

include: hydrogenated soy phosphatidylcholine (HSPC), 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine (DSPE), 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC), phosphatidylethanolamine (PE), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-di-(9-Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). The most commonly used synthetic PEG phospholipids are DPPE-mPEG-2000, DPPE-mPEG-5000, and DSPE-carboxy PEG-2000.

Liposomes over 400 nm in size have been shown to be rapidly cleared from the bloodstream upon systemic administration [110]. These particles are picked up rapidly by the RES and MPS. Opsonisation, being one of the most active pathways for liposomal destruction in the bloodstream, can be prevented significantly if the outer layer of these particles is functionalized with PEG chains. Such modifications stabilize the liposomes through steric effects and have been extensively discussed by Drummond and co-workers [111]. Steric stabilization of liposomes has shown to increase their longevity in circulation. Surface modification of liposomes is based on the concept that a hydrophilic polymer possessing a flexible chain, such as PEG, occupies the space immediately adjacent to the liposome surface and tends to exclude other macromolecules from this space [112]. As a result of this steric involvement, access and binding of blood plasma opsonins to the liposome surface are hindered, and thus interactions of MPS macrophages with such liposomes are inhibited [112].

Thus these modified nanoparticles exhibit long-circulating properties and significantly increase the circulation half-life of the particles compared with nonmodified liposomes. Longer circulation time directly correlates to a higher probability of the drug entrapped in particles reaching the target site. Doxil [PEGylated doxorubicin (DOX) liposomes] made by Ben Venue Laboratories for Johnson & Johnson (OH, USA) is one such stealth liposome that has been approved by the US FDA since 1997.

Liposomal formulations of several active molecules are currently in preclinical and clinical trials in different fields including cancer chemotherapy. One of the most interesting developments in this field is the potential of liposomes to combat the problem of multidrug resistance (MDR) of cancers cells that drastically reduces the efficacy of chemotherapeutic agents. In a recent study, Ogawara and co-workers, investigated the effect of PEG liposomal DOX in an orthotropic mouse tumor model inoculated with either colon cancer (C26) cells or their DOX-resistant (MDR) subclone, overexpressing P-glycoprotein efflux pumps [71]. The results showed that PEG liposomal DOX had anti-tumor effects on both DOX-resistant and nonDOX-resistant C26 cells.

Small-molecule ligands have also been evaluated as targeting agents for liposomal drug delivery. Bruckheimer and co-workers have demonstrated the anti-tumor efficacy of systemically administered, folate-targeted, lipid-protamine-DNA complexes (LPD-PEG-Folate) against breast cancer using an immunodeficient xenogenic murine model that has yielded promising results [73]. Some studies have investigated the effects of tumor targeting and gene delivery with antibody- or peptide-conjugated liposomes. In a

recent study, Rothdiener and co-workers, describe a liposomal siRNA [113] carrier system for targeted delivery of siRNA to CD33-positive acute myeloid leukemia cells. The particular siRNA is directed against the t(8:21) translocation resulting in the AML1/MTG8 fusion protein. In this study, the siRNA was encapsulated in free or polyethylene imine (PEI)-complexed form into PEGylated liposomes surface modified with an anti-CD33 single-chain Fv fragment for targeted delivery. Their results suggest that the siRNA-loaded immunolipoplexes (PILP) show specific binding and internalization by CD-33 expressing myeloid leukemia cell lines (SKNO-1, Kasumi-1). In another study, PEGylated immunoliposomes, a novel and efficient gene delivery system, have been developed by Hu and co-workers, by employing DNA–polyethylenimine (PEI 25 kDa) polyplexes, as well as anionic liposomes [72]. The CD-33 antibody is conjugated to these lipoplexes via streptavidin-monoclonal antibody conjugating through the biotin group located at the distal end of the PEG spacer as targeting antibody. These complexes have shown high efficiency in gene delivery to liver cancer cells with no significant cytotoxicity in *in vitro* studies. Intravenous administration of the PILP resulted in tumor and liver targeted gene expression of the reporter genes.

Targeting of liposomes using small peptides has also been explored to increase the efficacy of certain chemotherapeutic drugs. RGD modified DOX loaded liposomes have been evaluated by Zhang and co-workers for co-encapsulation and sequential release of antiangiogenic agent combretastatin A4 and DOX. *In vivo* experiments conducted in mice as an orthotopic model of B16F10 melanoma tumors demonstrated that RGD-modified liposomes exhibited the most pronounced tumor regression effect when both combretastatin A4 and DOX were co-encapsulated in addition to RGD targeting. *In vitro* release experiments also indicated that the rate of DOX was significantly slower than that of combretastatin A4.

1.2.5.3. Dendrimers. Dendrimers are spheroid or globular macromolecular nanostructures that are precisely engineered to carry molecules encapsulated in their interior void spaces or attached to the surface [114]. These systems have been extensively explored as solubilizing agents of hydrophobic drugs [115]. More than 100 compositionally different dendrimers have been designed and evaluated and show potential as drug delivery systems [116, 117]. Applications have included solubility enhancement [115], MRI contrast agents [118], gene therapy [119], drug delivery [120], and photodynamic therapy [121].

Structural symmetry around the core of the dendrimer is its most unique characteristic and these structures often adopt a spherical 3D morphology. In the absence of the dendrimer core, the dendrimeric structure can be further broken down to a number of identical fragments called dendrons. The resulting number of dendrons depends on the multiplicity of the central core ($n, n + 1, n + 2 \dots n + n'$). A dendron can be spatially divided into three different regions: the core, the interior (or branches) and the periphery (or end groups) (**Figure 1-10**). The number of branch points encountered upon moving outward from the core of the dendron to its periphery defines its generation (G-1, G-2 or G-3) [122]. Dendrimers of higher generations are larger, more branched and have more

Figure 1-10. Dendrimeric nanoparticle drug-delivery system

The dendrimeric structure is centered on a core anchor that is attached to a dendron via a linker. The distal atoms of the dendron act as further attachment points of structurally identical dendrons forming a regular branching structure. The generation of the dendrimer is indicated in the figure (G1, G2 and G3). Repeated branching of the dendrons ultimately leads to the formation of a spherical macromolecule that is sometimes referred to as a ‘starburst’ dendrimer. The external surface of these particles can be surface modified (A) with PEG chains to impart stealth properties; (B) by attaching antibodies as targeting ligands for active targeting; (C) by attaching a small molecule for active targeting; and (D) by exploiting the exterior surface charge (cationic) to complex DNA or siRNA. The drug can either be encapsulated in the voids between the branching structures or can be a structural part of the dendron.

end groups at their periphery than dendrimers of lower generations. They have dimensions in the range of 1 to over 10 nm; however the actual size of these systems is dependent on the generation of the dendrimer. Unlike micellar or liposomal systems, these molecules are synthetic macromolecules, and are generally form monodisperse systems.

Dendrimers can be synthesized from a variety of building blocks; the nature of the final dendrimeric nanoparticle dosage forms significantly depends on the solubility, biodegradability and biological activity of these building blocks. The most commonly used types of dendrimers in drug delivery include polyamidoamine [123], polyamines [124], and polypeptides [125] among others. Polyamidoamine (PAMAM) is the most common scaffold employed to study nanoparticle drug delivery and is commercially available in different size ranges and generations. Moreover these particles can be surface modified with targeting ligands thus offering an opportunity to have active as well as passive targeting. Active targeting has been explored with multiple targeting ligands; they include RGD [126], transferrin [127], folate ligands [88], and antibodies [93].

The control over size, shape, and surface functionality makes dendrimers one of the most customizable nanotechnologies commercially available. The properties of dendrimers are dominated by the functional groups on the molecular surface, however, there are examples of dendrimers with internal functionality [128] or therapeutic potential by itself [129]. Controlled formation of covalent dendrimeric structures leads to increases in diameter that is linear to the generation number, whereas the number of surface groups increases geometrically relative to the generation number. The dense, closely packed terminal groups of the outer surface of the dendrimer presents an ideal surface of molecular modification and has been exploited by researchers for drug delivery system approaches in oncology [130].

A number of specifically engineered as well as commercially available dendrimers have been explored for potential drug delivery systems. In a recent study, Rasines and co-workers, reported the synthesis of a new family of amine- and ammonium-terminated carbosilane dendrimers that undergoes quaternization with HCl or MeI. The resulting quaternized dendrimers have been shown to be soluble and stable in water or other protic solvents for long time periods [131]. This is a specific example of a water-soluble dendrimeric system that also possesses intrinsic therapeutic antibacterial activity. In another study, Santos and co-workers, developed systems with hydrophobic peripheral moieties accompanied by a hydrophilic interior; such a system shows good solubility in nonaqueous solvents [132]. Dendrimeric nanoparticles can be modified with hydrophilic PEG molecules to improve solubility of certain hydrophobic drugs. Ooya and co-workers have reported that graft and star-shaped graft polymers consisting of PEG400 graft chains increased the solubility of paclitaxel in water by three orders of magnitude. Polyglycerol dendrimers (dendriPGs) dissolved in water at high concentrations without significantly increasing the viscosity and, at 80% (w/w), were found to increase the solubility of paclitaxel 10,000-fold [89]. In another study, Bhadra and co-workers developed and evaluated the use of uncoated and PEGylated newer PAMAM dendrimers for delivery of anticancer drug 5-fluorouracil [90]. The results of this study suggest that

PEGylation of the system increased their drug loading capacity, reduced their drug release rate and hemolytic toxicity. In addition, such modifications also reduced drug leakage from the nanoparticle. Zhu and co-workers developed PEGylated PAMAM dendrimer conjugated to DOX and have evaluated it's *in vitro* and *in vivo* accumulation [91]. In this study, DOX was conjugated to differently PEGylated PAMAM dendrimers and *in vitro* evaluations including pH-dependent DOX release, cytotoxicity, cellular uptake, cell internalization mechanism and intracellular localization were performed. The results of the study indicated that DOX release from acid-sensitive dendrimer conjugates in a pH-dependant process and increased with increasing degree of PEGylation. *In vitro* cytotoxicity of these conjugates against ovarian cancer (SKOV-3) cells increased, while cellular uptake decreased with increasing degree of PEGylation. On the other hand the acid-insensitive dendrimer conjugates released negligible drug at any tested pH condition and were less cytotoxic. The authors also concluded that the conjugates were internalized by SKOV-3 cells via clathrin-mediated and adsorptive endocytosis. They subsequently were delivered to acidic lysosomes where DOX was released from acid-sensitive conjugates and diffused into the nuclei.

Extensive structural modifications of the dendrimer composition have also led to the development of novel small-molecule ligand targeted nanoparticulate drug delivery systems. Majoros and co-workers have developed unique PAMAM dendrimers functionalized with folic acid and methotrexate in *in vitro* and *in vivo* studies with promising results in *in vivo* animal models in retarding tumor growth [88]. In another study, Singh and co-workers, evaluated fourth generation PAMAM dendrimers conjugated to the surface primary amines to folic acid and folic acid-PEG (poly (ethylene glycol))-*N*-hydroxysuccinamide [133]. This system was evaluated in mice inoculated orthotopically with KB (human nasopharyngeal carcinoma) cells. These dendritic conjugates were loaded with 5-FU and administered in tumor-bearing mice. Results from the study indicated that the folate-PEG-dendrimer conjugate was significantly safe and effective in tumor targeting compared with a non-PEGylated formulation. Recently, functionalization of a second-generation triazine dendrimer surface modified with paclitaxel has been reported [92]. These dendrimers were further functionalized with 2 kDa PEG chains. *In vitro* testing employing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays indicated that these constructs exhibited low nanomolar activity on selected cell lines.

Functionalization and modification of targeted dendrimeric nanoparticles is not limited to small molecules. Recent advances in dendrimer chemistry have yielded a number of systems that can carry the payload of large biomolecules such as siRNA. One such example of targeted dendrimeric systems is exemplified by a study conducted by Waite and co-workers [126]. In this study, fifth generation PAMAM dendrimers were modified by the addition of cyclic RGD targeting peptides and were evaluated for their ability to associate with siRNAs. This system was evaluated against U87 glioma cell lines. The targeted construct was shown to associate itself to siRNA and form complexes that were approximately 200 nm in size. *In vitro* experiments indicated that the delivery of siRNA through 3D multicellular spheroids of U87 glioma cells was enhanced using PAMAM-RGD conjugates compared with the native PAMAM dendrimers. In a different

approach, Santos and co-workers developed PAMAM dendrimers that were decorated with hydrophobic alkyl chains on the outer dendrimeric surface in order to harness the advantages of interaction of cationic dendrimers with cells, as well as the capacity for lipids to interact with cell membranes [134]. It was hypothesized that these constructs have the ability to neutralize, bind and compact plasmid DNA (pDNA). Results of their study indicate that these new systems show a remarkable capacity for mediating the internalization of pDNA with minimum cytotoxicity. Ofeck and co-workers also developed novel polymerized PG-based dendrimer core shell structures to deliver siRNA to tumors *in vivo* [135]. The authors suggest that these cationic dendrimers can strongly improve the stability of the siRNA, its intracellular trafficking, its silencing efficacy and its accumulation in the tumor environment owing to the enhanced permeability and retention effect. These systems were evaluated in *in vitro* models of human glioblastoma and murine mammary adenocarcinoma cell lines with promising outcomes. Polyplexes formed by the interaction of plasmid DNA and cationic dendrimers have also been extensively explored. Hyperbranched polyether polyols have been partially functionalized with quaternary or tertiary ammonium groups by Tziveleka and co-workers resulting in dendritic polymers that interact with pDNA affording polyplexes for gene delivery [136].

1.2.5.4. Nanoemulsions. Nanoemulsions are fine multiphasic systems generally comprising of an oil-in-water dispersion. These nanoparticles are less than 100 nm in size; however larger particles between the size ranges of 100 to 600 nm have also been reported (**Figure 1-11**) [137]. The dispersed medium, or the continuous medium, in such systems is aqueous, whereas the dispersed phase is comprised of a nonaqueous phase such as oils, triglycerides (Miglyol®, Myritol®) or lipids (Lipoid E8®). The system is stabilized by using surfactants at lower concentrations than those used for micro-emulsion or coarse emulsions.

Nanoemulsions have been explored as candidates for drug delivery systems because of advantages such as improved drug solubilization, long shelf life, and ease of preparation and administration. These systems can be manipulated constitutively to reach a target site and have been explored for controlled release and targeted delivery of different pharmaceutical agents. One major disadvantage of this system is that these nanoparticles are in a metastable state; they are not thermodynamically stable and are only kinetically stable thus making them a very fragile system. With this in mind, nanoemulsions have gained a lot of attention in applications for topical administration. There have been attempts to use these systems for targeted drug delivery for cancer chemotherapy.

Tagne and co-workers have recently compared the physicochemical characteristics of dacarbazine suspension to dacarbazine nanoemulsions [74]. The group also evaluated the therapeutics benefits of dacarbazine nanoemulsion in an orthotopic model of melanoma in mice. Surface charge studies on the nanoparticles indicated that the nanoemulsion preparation demonstrated a lower ζ -potential relative to suspensions of dacarbazine. The authors also report that, relative to suspensions of dacarbazine with a

Figure 1-11. Nanoemulsion

The surfactant encapsulates a hydrophobic liquid droplet. These systems are usually oil-in-water emulsions that are thermodynamically less stable than other systems. The hydrophobic drug can be dissolved in the oil droplet that is stabilized by the surfactant in an aqueous environment.

mean particle size of 5470 nm, nanoemulsions of the same drug showed mean particle sizes of 131 nm and were more efficacious. The animal studies indicated that topical application of nanoemulsions compared with the suspension preparation produced up to tenfold greater percentage reductions of tumor size. The nanoemulsion therapy when compared with the suspension preparations, showed fivefold greater efficacies (73% versus 14%) in preventing tumor growth in live animals. In another related study, the authors also reported the preparation and evaluation of tamoxifen loaded nanoemulsions having a particle size of approximately 47 nm. This system inhibited cell proliferation 20-fold greater and increased cell apoptosis fourfold greater in the HTB-20 breast cancer cell line [75].

The highly potent antineoplastic drug camptothecin (CA) has low aqueous solubility and limited availability at the tumor site. Han and co-workers have developed an oil/water nanoemulsion to enhance the solubility, stability and antitumor activity of a novel 10-methoxy-9-nitrocamptothecin (antineoplastic drug and CA derivative) [78]. A nanoemulsion of the drug was prepared using Lipoid E80 and Cremophor® EL as main emulsifiers by microfluidization. The researchers reported that the drug was incorporated in the nanoemulsion in the form of lactone with a concentration of 489 µg/ml, more than 200-fold higher than that in water. The nanoemulsion showed a marked increase in cytotoxic activity of the drug (23.6-fold and 28.6-fold in S180 cells and A549 cells, respectively) via arresting the cell at G2 phase.

The effect of surface modification of these nanosystems with small molecule ligands has been explored by some groups and has been reported in the literature. Ohguchi and co-workers recently published a study in which selective delivery of a folate-PEG-linked nanoemulsion loaded with aclocinomyacin A to nasopharyngeal tumor cells was achieved [77]. Folate linked, nanoemulsion loaded systems were formulated with folate-PEG5000-DSPE, and folate-PEG5000-DSPE. The folate-PEG5000 linked nanoemulsions showed a 3.3-fold greater association with tumor cells compared with the nonfolate nanoemulsion.

1.2.5.5. Solid lipid nanoparticles. Solid lipid nanoparticles are solid particulate constructs that constitute a colloidal system composed of a biocompatible/biodegradable lipid matrix that exists as a solid at body temperature. These particles exhibit size range between 100 and 400 nm (**Figure 1-12**). Since their conceptual realization in the early 1990s [138], a number of researchers have explored the potential of these systems to act as an effective chemotherapeutic drug delivery system. This is partly due to the fact that these systems can be used as efficient alternative lipophilic colloidal systems that are biocompatible, biodegradable and use common pharmaceutical excipients. Solid lipid nanoparticles (SLNs) combine advantages of other colloidal drug carrier systems such as liposomes, polymeric nanoparticles and dendrimers, but at the same time avoid or minimize certain drawbacks associated with them. One of the major advantages of using SLN as potential drug delivery system is the aspect of controlled release of the incorporated drug within the particulate matrix. Generally the drug is gradually released from the particles through a process of erosion initiated by enzymatic degradation. The

Figure 1-12. Solid lipid nanoparticle

Structurally these are solid lipid spheres in the nanometer range that constitute a lipid matrix. Hydrophobic drugs or drugs that need to be encapsulated can be entrapped in this matrix. Surface modification can aid in imparting stealth properties, for example, introduction of a long-chain PEG on the exterior surface. This structural feature aids in the attachment of small molecular targeting agents on the surface for active targeting. Depending on the constituents and the surface modification approaches, researchers have been able to obtain a cationic layer on the exterior; **(A)** this can help in delivery of nuclear material such as siRNAs and DNA.

rate of release may be controlled by the nature of the lipid used in the construction [139], particles size of the colloidal particles, and choice of surfactant [140] and also by the inner structure of the SLNs [141]. These systems exhibit excellent physical stability, protection of incorporated labile drugs, good tolerability and surface modifications leading to site-specific targeting. The potential disadvantages include insufficient loading capacity, drug expulsion after polymeric transition during storage and relatively high water content of the dispersions [142] [143].

Although no SLN parenteral product has been approved as a commercial product, intensive research on SLNs has been reported [138]. SLNs may be injected intravenously and used to target chemotherapeutic agents to diseased organs. However, these particles are also rapidly cleared by the RES and MPS. Thus to facilitate drug targeting in tumor tissue, surface modification can be carried out to impart stealth characteristics. Generally, polyoxyethylene–polypropylene copolymers, such as Pluronic F188 can be used. In such a system the hydrophobic portion of the polymeric molecule forms the nanoparticle matrix, while the water-soluble polyoxyethylene block forms a hydrophilic coating on the particle. Chen and co-workers have developed ‘stealth’ SLNs that have the ability to increase accumulation of drug loaded SLNs in tumors [81]. In the study they investigated the body distribution of an anti-tumor drug CA-loaded SLN (CA-SLN) with an average particle size of 197 nm intravenously administered to mice. Pharmacokinetic studies showed better bio-distribution of CA-SLN (18-fold enhancement) compared with CA solution. This was accompanied by accumulation, especially in brain, heart and reticuloendothelial cells containing organs. The authors attribute this efficacy to their small size or association of SLN with lipid bilayers of red blood cells [144]. These nanoparticles have also showed significant potential in delivering antineoplastic drugs to the brain and aid in crossing the blood–brain barrier. Similar studies with respect to pharmacokinetic parameters of stealthy SLNs have been reported by other groups with promising results [139].

In another study the pharmacokinetics and tissue distribution of DOX incorporated in non-stealth SLNs and in stealth solid lipid nanoparticles (SSLN), containing stearic acid–PEG 2000 as stealth agent, after intravenous administration to conscious rabbits, was assessed by Zara and co-workers [82]. Results from their study showed that DOX AUC increased as a function of the amount of stealth agent present in the SLN. The drug was detectable in the blood 6 hours after the injection of SLN or stealth SLN, while no DOX was detectable after the IV injection of the commercial doxorubicin solution. Tissue distribution of doxorubicin determined at different time points after the administration showed presence of DOX in the brain only after the SLN and stealth SLN administration.

Targeting of these nanoparticles via surface modification is a developing field and many groups have attempted to achieve tumor site specificity using targeting ligands. One such study was performed by Oyewumi and co-workers, wherein gadolinium SLNs were coated with folic acid and PEG 3350 [83]. SLNs were engineered from oil-in-water microemulsion templates using emulsifying wax and gadolinium hexanedone as the oil phase. To obtain folate-coated nanoparticles, folate ligands and folic acid chemically

linked to DSPE via a PEG spacer (MW 3350), were included in nanoparticle preparations. These systems were tested in the *in vivo* model of KB tumor xenografted in athymic mice. The results of their study indicated that, both folate-coated and PEG-coated nanoparticles had comparable tumor accumulation. However, the cell uptake and tumor retention of folate-coated nanoparticles was significantly enhanced over PEG-coated nanoparticles. Ferritin-coupled solid lipid nanoparticles were investigated for tumor targeting. In a recent study, Jain and co-workers reported the development of 5-FU bearing ferritin appended solid lipid nanoparticles for tumor targeting [145]. SLNs were prepared using HSPC, cholesterol, DSPE and triolien. The average size of SLNs was found to be in the range 110–152 nm. *In vitro* studies indicated that the binding of ferritin–SLN exhibits 7.7-fold higher binding to MDA-MB-468 breast cancer cells in comparison to unfunctionalized SLNs; administration of ferritin–SLN formulation results in effective reduction in tumor growth in *in vivo* experiments as compared with free 5-FU and plain SLNs. Another active field of research is the design and preparation of cationic SLNs and their targeting to alveolar macrophages [80, 146]. These particles express charge on their outer surface and can efficiently bind DNA directly via ionic interaction and mediate gene transfection. In a study conducted by Yu and co-workers, mannan based PE-grafted ligand was synthesized and used for the surface modification of DNA-loaded cationic SLN to prepare Man–SLN–DNA. The results of their study indicated that Man–SLN–DNA produced the highest gene expressions when compared with nonmodified SLN–DNA and Lipofectamine 2000–DNA [80].

1.2.5.6. Carbon fullerenes. Fullerenes are macromolecular structures completely made of carbon, in the form of a hollow sphere, tube or ellipsoid. The first fullerene was synthesized in 1985 by Smalley and co-workers and was named ‘buckminsterfullerene’ or ‘buckyballs’, named after the architect Richard Buckminster Fuller, resembling his architectural geodesic domes [147]. Structurally the buckyball molecule is spherical in shape with 60 carbon atoms (C_{60}) arranged in interlocking hexagons and pentagons (**Figure 1-13**).

C_{60} (buckyball/buckminsterfullerene) is the smallest fullerene molecule in which no two pentagons are connected with a common edge. It resembles a soccer ball consisting of 20 hexagonal and 12 pentagonal rings, with a truncated polyhedral structure. Each carbon atom is bonded to three other carbon atoms and is sp^2 hybridized. The C_{60} fullerene contains two bond lengths; bonds between two hexagons, which are shorter and consist of double bonds; and bonds between a hexagon and pentagon. The earliest bulk production process of C_{60} and C_{70} fullerenes was developed in 1990 by Kratschmer and co-workers by electrical arc vaporization of graphite. These carbon-based systems have been the subject of intense research in the field of material science, electronics and nanotechnology.

Since these systems are built on a carbon backbone, they are inherently hydrophobic and are marred by solubility problems. As a result of this some researchers have raised concerns over the potential of these systems as effective drug delivery vectors [148]. Toxicity issues associated with these constructs have also undermined their initial

Figure 1-13. Carbon buckyball

These are macromolecular structures with precise structural feature made up of sp^2 carbons. The hydrophobic interior can entrap hydrophobic drugs, whereas the carbon skeleton can help in diversifying the external surfaces. Primarily hydrophobic in nature, these particles can be surface modified to have PEG chains, functional groups and linkers such as carboxylate ($-COOH$) groups, and amine ($-NHR$) linkers. Drug molecules can also be attached on the exterior surface with biodegradable linkers to achieve controlled release of drug.

development [149]. However other advantages of these systems such as their size, three-dimensionality and electronic configurations make them an appealing material for drug delivery. The customizable carbon skeleton structure accompanied by the scope for surface modification makes them attractive dosage form agents.

In order to improve the solubility of these systems extensive research interest in developing newer water soluble constructs has yielded interesting results [150]. Ashcroft and coworkers have recently reported the design and preparation of water-soluble C₆₀ derivatives can cross cell membranes and even produce transfection [151]. Specifically the authors of this study report the synthesis and characterization of the C₆₀ fullerene covalently attached to proteins as a first step in evaluating fullerene immunotherapeutic systems. In another study, Lu and co-workers reported an aqueous fullerene C₆₀–DOX conjugate system that is formed through amide linkage. This system was shown to preserve the intrinsic properties of DOX and the structural feature of the fullerene cage. In their *in vitro* studies on human breast cancer cell lines, these conjugates with hydrophilic ethylene glycol spacers showed superior aqueous compatibility. The results also indicated that the antineoplastic activity of DOX was unaffected as a conjugate. In a recent study, Zhu and co-workers have reported the preparation and characterization of a new multihydroxylated methanofullerene carboxylic acid derivative with high aqueous solubility. The authors indicate that this system exhibits low cytotoxicity in the *in vitro* MTT assay.

Carbon nanotubes (CNT) are another type of carbon skeleton based macromolecular structure that have gained considerable attention as drug delivery systems. Fullerene related nanotubes were discovered by Sumio Iijima in 1991 using a simple arc-evaporation method [152]. The word nanotube is derived from their size, as the diameter of a nanotube is in the order of a few nanometers and the length can be up to several micrometers. CNT is also known as “buckytube”, and is a member of the fullerene family, which are composed of sp² bonds that are held together with van der Waals forces of attraction. These are cylindrical in shape and exist as single-walled carbon nanotubes (SWCNTs) or multiwalled carbon nanotubes (MWCNTs) (**Figure 1-14**). SWCNTs have a diameter of approximately 1 nm and the length may be several thousand times larger (centimeters). These structures can be structurally conceptualized as a one-atom-thick layer of graphite (graphene) wrapped into a seamless cylinder. On the other hand, MWCNTs are produced with multiple layers of graphite rolled on in a concentric configuration. The distance between the layers is approximately Å and outer diameter could range from 1 to 50 nm. These macromolecular structures can be manufactured in sizeable quantities employing techniques such as carbon arc-discharge, laser ablation, high-pressure carbon monoxide and chemical vapor deposition.

Carbon nanotubes have been evaluated as potential drug-delivery system due to their numerous advantages. Some of these advantageous parameters are directly related to their physicochemical characteristics such as enhanced cell membrane penetrability, high surface area and the ability to surface modify these constructs in a reproducible and quantitative manner. Functionalized hydrophilic CNTs exhibit superior biocompatibility but are susceptible to opsonization by the MPS and RES leading to lower retention time

Figure 1-14. Single-walled carbon nanotubes

These are tube-like macromolecular structures with precise structural feature made up on sp² carbons. Like buckyballs, these systems have hydrophobic interior that can entrap hydrophobic drugs, whereas the carbon skeleton can help in diversifying the external surfaces. Carbon nanotubes are also primarily hydrophobic in nature and can be surface-modified to have PEG chains, functional groups and linkers such as carboxylate (-COOH) groups, and amine (-NHR) linkers. Active targeting using antibodies has also been explored. Drug molecules can also be attached on the exterior surface with biodegradable linkers to achieve controlled release of drug. Multi walled carbon nanotubes also share the same structural features but have multiple layers of tubes arranged in a concentric manner.

in the bloodstream. In order to avoid this problem, PEGylation has been employed to resist opsonization and to achieve long circulation *in vivo* [[153-155]. However, if the PEG chain is long, it has been shown to accelerate complement activation and lead to weak cellular uptake in spite of their longer circulation in the blood stream [84, 85]. Thus, it is important to balance such contradictory factors to achieve effective CNT based carriers. Researchers have demonstrated that adsorbing phospholipid-PEG 2000) PL-PEG-2000 to SWNTs does not prevent uptake of SWNTs *in vitro* [85] nor *in vivo* [84] while functionalizing with longer chain PEG (PL-PEG5000) reduced their nonspecific uptake by cells *in vivo*. The overall performance of the conjugate also depends up on the linker tethering the drug to CNTs.

A number of anti-tumor drugs linked to CNTs through covalent or noncovalent interaction have been designed and evaluated as potential drug delivery systems.

In a recent study Bhirde and co-workers combined both cisplatin and epidermal growth factor with SWCNTs through the coordination and amidation of nanotube bound carboxylic groups, respectively, to treat squamous cell cancer [156]. The results showed excellent *in vitro* and *in vivo* antitumor activity in nude athymic mice orthotopically innnolated with HN13 cells. In another study, Wu and co-workers recently reported a MWCNT based drug delivery system that was developed to deliver 10-hydroxycamptothecin (HCPT) [86]. The authors have designed a system in which covalently binding CNTs through cleavable ester linkages were conjugated to HCPT employing diaminotriethylene glycol as the spacer between the drug and the nanotube. They reported that this conjugate system showed superior antitumor activity, both *in vitro* and *in vivo* when compared with clinical HCPT formulation. The MWCNT-HCPT conjugates showed longer blood circulation and high drug accumulation at the tumor site. A folic-acid-targeted SWCNTs drug delivery system, which can be triggered by changes in pH and loaded with DOX, has been evaluated by Zhang and co-workers [87]. The SWCNTs in this study were derivatized with carboxylate groups and coated with a polysaccharide. The drug, DOX, was the reported to be in a bound state in this system at physiological pH and dissociated at a lower pH. The drug-loaded system was surface modified with folic acid as a targeting ligand and the system was evaluated against HeLa cells. The results of their study indicated that the system caused damage in the nuclear DNA and inhibited cell proliferation.

In a novel approach Radu and co-workers recently attempted to exploit the thermal characteristic so CNTs [157]. In their proof-of-concept study they conjugated CD-22 monoclonal antibodies the SWCNTs to target acute lymphoblastic leukemia cells *in vitro*. In their study the authors treated CD-22-expressing Daudi cells with CD-22-antibody-conjugated SWCNT followed by exposure to near infrared light at 805 to 811 nm. Upon exposure to these conditions, the authors reported that they were successful in killing the cancer cells by the novel photo-ablation therapy.

1.2.5.7. Emerging nanoparticle systems. A number of new nanoparticulate drug delivery systems are currently being evaluated for delivery of chemotherapeutic

agents. Some of these systems are fairly new. ‘Nanocells’, one such relatively new system, have been developed by Sengupta and co-workers [79]. The authors report that they have designed this novel delivery system, comprising a nanoscale PEGylated phospholipid block-copolymer envelope coating a central nanoparticle. The authors then proceeded to conjugate a chemotherapeutic agent to the nanoparticle and an antiangiogenic agent was trapped within the lipid envelope. The construct, termed as a ‘nanocell’, has shown sequential release of the two drugs associated with the system. The study indicated that the outer envelope containing the antiangiogenic drug was released first, impairing the vascular flow followed by the release of the drug (DOX) from the inner nanoparticle. The authors hypothesized that once the vascular supply was impaired the nanoparticles would be trapped inside the tumor and in a second step release DOX.

Another interesting approach has been evaluated by Al-Jamal and co-workers, where they reported a novel cationic lipidic-polylysine dendron that self-assembled in water, forming unusual small vesicular structures, which they termed as ‘dendrosomes’ [158]. In this proof-of-concept study they were able to encapsulate the drug benzyl penicillin (penicillin G) as a model of an orally labile, negatively charged water-soluble antibiotic. In a related study they reported that dendrosomes of different compositions had high encapsulation efficiencies and slower release rates. Inclusion of cholesterol was found both to increase the size of the aggregates from around 310 to 560 nm and to increase shape irregularities [159]. Dutta and co-workers recently reported a study where the potential of dendrosomes for the delivery of siRNA targeting E6 and E7 proteins of cervical cancer cells *in vitro* was explored [160]. For cervical cancer the E6 and E7 oncogenes are ideal siRNA targets for treatment. In their formulation the siRNA was encapsulated into dendrosomes to mask its toxic effects. The optimized dendrosomal formulation was found to possess an entrapment efficiency of $49.76 \pm 1.62\%$, vesicle size of 154 ± 1.73 nm, and zeta-potential of $+3.21 \pm 0.07$ mV. Zakharian and co-workers recently reported a hybrid fullerene-paclitaxel conjugate that has been synthesized as a slow-release drug for aerosol liposome delivery of paclitaxel for lung cancer therapy [161]. The conjugate was designed to release the drug via enzymatic hydrolysis and subsequently has shown a half-life of release of 80 min in bovine plasma.

1.2.6. Future perspective

Recent advances in the design of nanoparticle delivery systems show great potential for overcoming the obstacles that prevent efficient drug delivery to solid tumors. These systems also provide the possibility of resolving limiting factors such as drug resistance and aid the transport of drugs across physiological barriers. Extensive research leading to the design and development of novel biomaterials that are biodegradable and biocompatible has hastened the development of nanoparticulate drug delivery systems. The possibility of targeting drugs selectively to diseased cells and sparing healthy tissue can further increase the efficiency of these systems by preventing premature degradation, adverse side effects, low doses of drug being administered, and also increase patient compliance.

Although development for cancer chemotherapy using small molecule or biological drugs will continue to be the primary mode of treatment, nanoparticulate drug delivery systems will offer an additional assistance to combat cancer. Nanoparticle research will not only continue to expand and help design effective drug delivery systems for newer drug candidates, but also help rejuvenate older chemotherapeutic agents that are effective but suffer from issues of drug resistance and side effects. In order to use these systems as a viable option for chemotherapy, extensive research has to be conducted with regards to their safety and stability. Another aspect that is usually neglected is the scale-up and manufacturing of these systems. These are complex systems that are often expensive and can be easily manufactured in small settings but are sometimes difficult to scale up. Effort has to be put into the development of new scale-up technologies that can manufacture these highly specialized systems in a reproducible and validated manufacturing process.

1.3. Iron Oxide Nanoparticles

The last decade has witnessed development of nanoparticulate technology in terms of fabrication, characterization and more importantly tailor made functional properties for application in biomedical and diagnostics field [32, 162, 163]. Over the last four decades smaller iron oxide particles are widely used in *in vitro* diagnostic agents. But in the last decade the evolution of variety of iron oxide nanoparticles (IONP) has taken place. Most widely used iron oxides in nanomedicine are maghemite ($\gamma\text{-Fe}_2\text{O}_3$) and magnetite (Fe_3O_4) of $\sim 5\text{-}20$ nm in diameter. Among those magnetite is the most favorable iron oxide due to its already verified biocompatibility. These IONP can be surface modified with variety of coatings for its dispersability in appropriate solvent to form a homogeneous suspension known as ferro-fluids [164]. These suspensions has a capability of interacting with external magnetic field for positioning to a determined site in the body for medical diagnosis through MRI and for cancer therapy with AC magnetic field [165]. See **Figure 1-15** for MRI imaging of rat brains after treatment with IONP [166].

Due to their small nano size, the IONP possess large surface area and quantum effects that guide the magnetic properties, which offers superparamagnetic phenomena. As each particle is considered as a single magnetic domain, they also exhibit quantum tunneling of magnetization [167]. The superparamagnetic nanoparticles possess distinctive properties such as mesoscopic physical, chemical, mechanical, and thermal properties, which offers variety of biomedical applications including:

- drug delivery [168]
- MRI [169, 170]
- hyperthermia [171]
- tissue repair [32]
- cell sorting and tracking [172]

Iron oxides has long safe history for clinical application. In particular polymer-

Figure 1-15. MRI images of IONP treated rat brains

Reprinted by permission from Macmillan Publishers Ltd: [Journal of Cerebral Blood Flow & Metabolism] (Liu, H.-L., et al., *In vivo assessment of macrophage CNS infiltration during disruption of the blood–brain barrier with focused ultrasound: a magnetic resonance imaging study*. Journal of Cerebral Blood Flow & Metabolism, 2010. **30**(1): p. 177-186.), copyright (2010). Top panel not treated with IONP, whereas bottom panel treated with IONP 24 hours before sonication. Images were acquired at different time points. These were imaged to observe the occurrence of an inflammatory response, which was showed (bottom panel) by the continuous expansion of the region (white arrows). This phenomenon was due to the accumulation of IONP-laden macrophages causing a high image susceptibility effect.

coated IONP have enough history for treatment of anemia in an injectable form. Examples include paramagnetic dextran-coated IONP are INFeD® (Watson Pharmaceuticals) [173, 174], Dexferrum® (Luitpold Pharmaceuticals) [174] and Feraheme (a.k.a. ferumoxytol, AMAG pharmaceuticals [175].

Superparamagnetic polymer-coated IONP have the tendency to lower the T2 contrast signal for MRI to efficiently distinguish healthy and pathological tissue. There are several FDA-approved IONP MRI diagnostic agents including Feridex IV (formerly supplied by AMAG Pharmaceuticals, recently discontinued) [176, 177] and Resovist (a.k.a. ferucarbotran, Bayer Schering Pharma AG) [178]. Though IONP have been used for various biomedical applications, in recent years there is significant attention towards the development of multifunctional IONP with complimentary roles [179]. For instance, the imaging improvement property of IONP can be used in combination with drug delivery applications to generate theranostics. This approach offers both real-time monitoring of drug delivery system to the target pathological tissue and to follow the therapeutic efficacy of the disease state [180]. The other nanoparticulate drug delivery systems including liposomes, micelles, and polymeric nanoparticles does not contain imaging property within the delivery system to follow the *in vivo* distribution process [177]. This implicates that IONP can also be used as vehicles for delivering therapeutic agents. Additionally these particles can be multifunctional that contains ligands for targeting as well as therapeutic agents. Targeting offers more focused delivery of the payload that results in less drug required to fall into the therapeutic window. Less drug will offer less toxic side effects.

1.3.1. IONP biocompatibility and toxicity

IONP are considered relatively safe with lack of toxicity for their biomedical applications [32]. There are different types of IONP used for human application and they exhibit different fate in the body depending on the type of core and coating material used. Their safe clinical application is already proven through standard pharmacological and toxicological examinations.

The human immune system consists of series of methods to remove foreign particulates from the circulation system. This could pose a challenge for engineering a long-circulating nanoparticulate drug delivery system. The RES (also known as the mononuclear phagocyte system) plays a crucial role in recognizing a foreign particulate material and then binding to opsonins (plasma proteins), which then cleared from the system. These opsonins have tendency to tag the foreign material to recognize as something that needs to be cleared through phagocytic cells like monocytes and macrophages. This process of reorganization of foreign particles, adsorption of opsonins and type of clearance from circulation system are influenced by physicochemical properties of nanoparticles like size and surface chemistry [181]. The phagocytic cells accumulate in organs mainly the kupffer cells in the liver and other macrophage-rich organs will regulate the biodistribution of iron oxides upon injection into the body.

IONP are coated with polymers to improve the physical stability. These polymers such as dextran are degraded by intracellular dextranases and then eliminated in the urine [182]. Similar degradation and elimination pattern was found with other biodegradable polymers used for coating. **Table 1-2** [32] summarizes a list of materials that can be used to coat the nanoparticles for stabilization and for varied biomedical applications. Whereas the iron from the nanoparticles enters the body's natural iron metabolic system, which is then sequestered in ferritin (iron storage protein). Which will finally ends up in the hemoglobin in red blood cells [181]. Even though iron is considered essential nutrient, it could cause toxicity sometimes when very large amounts of iron administered.

1.3.2. Synthesis of IONP

Ideally, the manufacturing process for IONP should be scalable to an industrial size, reproducible from batch to batch and yield in monodisperse IONP. The method of preparation will affect the physicochemical properties (e.g. size, shape, etc.) of the particles that could influence the magnetic property for MRI purpose. The monodispersity of the IONP will offer several advantages including easy to characterize, easier to control the properties, and more likely will in the favor of FDA approval. There are many methods available to produce surface modified IONP of desired size and shape. Electron beam lithography and gas phase deposition are considered physical methods, which are extensive and hard to manufacture techniques [183-186]. Additionally with these methods it's hard to control the particles size in nanometers and incapable to produce monodisperse particles. Whereas wet chemical methods offers better control over size, composition, shape and will produce monodisperse particles. In addition these process are more efficient, more tractable and easy to manufacture IONP [32]. Iron oxides such as maghemite ($\gamma\text{-Fe}_2\text{O}_3$) and magnetite (Fe_3O_4) can be synthetically produced by co-precipitation of Fe^{2+} and Fe^{3+} aqueous salts solutions with addition of base [187]. The size, shape, and composition of IONP can be controlled by the stoichiometric ratio of Fe^{2+} and Fe^{3+} , pH, ionic strength of the media, and salt type used such as sulphates, nitrates, and chlorides. The overall chemical reaction to obtain Fe_3O_4 is represented in **Equation 1-1**.

This reaction is favorable at pH between 8 and 14 with a stoichiometric ration of 2:1 of Fe^{3+} and Fe^{2+} in non-oxidizing oxygen free environmental conditions. Otherwise magnetite is unstable and easily gets oxidized to maghemite in the presence of oxygen as shown in **Equation 1-2**.

In order to avoid this oxidation process and also agglomeration typically magnetite nanoparticles are coated with organic or inorganic molecules during the precipitation itself as derived in **Equation 1-1** [32].

Table 1-2. Materials and its applications used for IONP coating

Material	Applications
Chitosan	Hydrophilic and biocompatible
Dextran,	Improves the colloidal stability and increases the blood
polyvinylpyrrolidone	circulation time
Fatty acids	Improves colloidal stability and aqueous dispersibility
Gelatin	Natural polymer, biocompatible, gelling agent, hydrophilic, and emulsifier
PEG	Improves the biocompatibility, increases blood circulation time and internalization efficiency of the IONP
Poly (D, L- lactide)	Biocompatible and low cytotoxic
Polyacrylic acid	Biocompatible and improves colloidal stability and bio-dhesion
Polypeptides	Helps to target cells
Polyvinyl alcohol	Improves stability by preventing aggregation of particles

The biomedical applications of IONP will improve our understanding of biological processes at the nanoscale. Although IONP have a long history for variety of applications in medicine and other fields, this dissertation will focus on their development to diagnose and treat disease in the field of nanomedicine for targeted drug delivery to IFIs.

1.4. Routes of Administration

For life threatening infections, the standard drug treatment strategy is to get as much antimicrobial drug as possible to the pathogen as fast as possible to obtain an effective pathogen kill rate. Therefore the route of drug administration is often an injectable true solution because oral administration is often variable in sick patients or physical chemical properties of drug product simply to not allow acceptable bioavailability [6, 12]. In critical patients, intravenous administration with an initial bolus dose followed by an infusion to maintain killing concentrations is often the norm [1]. The assumption of this approach is that if sufficient concentrations of the drug are maintained in the blood stream enough of the antimicrobial agent is being “pushed” to the infection site to produce an adequate kill rate.

In contrast, particulate nanomedicines are designed to overcome specific problems in drug delivery. For example, amphotericin B solution has significant renal toxicity and administration problems [188]. Most toxicities are dose related. If killing effectiveness can be raised, the total dose administered can be cut, hopefully avoiding or ameliorating some of the toxicity. In previous studies from our laboratories with highly toxic anticancer agents, formulating the drug into a nanomedicine increased killing efficiency and allowed for a 20%-30% reduction as compared to the non nanomedicine formulations [26]. Several properties contribute to this effect. First, with nanomedicines the dosage form can be targeted to the site of action either passively or actively (or both simultaneously) so that the drug substance is not diluted throughout the body to tissues that do not need the drug [24, 189]. For nanomedicines, the blood concentration may not be indicative of the concentration needed for treatment. Rather the drug substance concentration at the intended site is more important. Second, nanomedicines can be optimized so the drug has an extended release profile locally over several days. Third, a diagnostic agent in the nanomedicine particles should allow for a more accurate diagnosis as to the spread of the infection [190]. Hopefully, monitoring of subsequent doses will also allow serial image monitoring of the recovery process. This should allow the attending physicians to better monitor and change dosing regimens thereby producing a more controlled therapeutic response. Fourth, nanomedicine particulates often avoid molecule focused active transport body systems that eliminate drugs in true solution from the body [189, 191]. Drug resistance by this mechanism is a fairly common problem with some critical drugs and is not easily overcome. However, most drug extransporters work on molecular drugs in true solution, not on significantly larger particulate nanomedicines. In this dissertation, the drug particles are approximately 30 nm and too large for molecular transporters.

With colloidal nanomedicines, size and surface charge matters. Usually internal nanomedicines for parenteral use are in the range of 200 nm or smaller [24, 191]. As size decreases effectiveness can increase. Part of this is due to vascular permeability, Biological barriers such as vasculature become much more porous when inflammation is present. This allows small nanomedicine particles to pass onto tissues and have significant penetration to the infection site. This allows for potential intravenous, intramuscular and subcutaneous routes of administration. Since invasive fungal infections include infections of the CNS and lungs, the delivery system properties are important. With fungal pneumonias it may be possible to administer nanomedicine particles by nebulization both as a nontargeted and target product. Concurrent intravenous administration may also be warranted.

1.5. Scale Up and Manufacturing Issues

Generally, pharmaceutical manufacturing is done as a batch process rather than a continuous process. A potential product is conceived and design specifications are set. Next individual unit operation steps are decided and placed in a specific order to produce the product. Eventually each step is optimized and batches are produced for testing and setting specifications. The small scale laboratory or manufacturing process is tested for reproducibility and validated for small batches. For sterile products the process is more complicated because of additional quality requirements and specifications. Advancing from laboratory scale to making batches large enough to meet animal testing needs and eventually early clinical trials require significant funds, facilities, personnel and expertise. Also during processing design and scale up there may be only limited amounts of the active pharmaceutical ingredient and obtaining cGMP manufactured drug substance can be a significant issue. Usually dosage form manufacturing processes that are less complicated (less steps, more available equipment, more reproducible) are less expensive and developed faster to produce test batches. This helps to expedite the drug product development process and facilitates required studies such as drug product stability studies.

1.6. Central Hypothesis and Specific Aims

1.6.1. Introduction

IFIs are associated with high morbidity and mortality, particularly in immunocompromised hosts who are at high risk for these infections. The mortality rates of some of these IFIs go beyond 60% in certain situations, and are one of the greatest challenges facing clinicians who care for immunocompromised patients [8]. Patients with cancer chemotherapy, HIV infection, and acute leukaemia or haematopoietic stem cell transplant receivers are at high risk for IFIs. The incidence of IFIs is further increased with aggressive treatment strategies, together with improvements in medical care that extend survival of critically ill patients. Thus, leading to increased number of

immunocompromised patients [192]. Additionally, an increasing variety of fungal species has been found, particularly in these patients [5, 193, 194]. One of the most striking estimates recently was the global burden for cryptococcal meningitis in HIV infected patients with about one million cases each year. It is estimated that 625,000 deaths occur among these 1M cases every year [2].

To date no ideal antifungal agent exists to cure IFIs [8]. Older agents are toxic and newer agents including azoles and echinocandins come with varied pharmacokinetic profiles, drug interactions, a narrow spectrum of activity and limited routes of administration, thus limiting their application [8]. AMB was discovered in 1955 and it is still considered a gold standard for treatment of IFIs since its FDA approval in 1959. It has broadest spectrum of activity against species of fungi including *Candida* species, *Cryptococcus neoformans*, and *Coccidioides immitis* [9]. The conventional formulation, AMB-D is associated with a high incidence of infusion-related adverse effects and dose-related nephrotoxicity, thus limiting its usage. To address this problem lipid formulations of AMB have been developed, but the relative efficacy and safety of these drugs has not been adequately studied [8, 192]. Despite several advancements in antifungal therapy, the best therapy of IFIs still remains a challenge. The development of new anti-fungal drugs for the treatment of IFIs has not kept pace with the progress in fungal therapy, because of the nonspecific drug distribution resulting in low fungal concentrations and systemic toxicity.

The effectiveness of the fungal treatment is directly related to the treatment's ability to target and to kill the fungal cells while affecting as few healthy cells as possible. The degree of change in the patient's quality of life and eventual life expectancy is directly related to this targeting ability of the antifungal treatment. The advances in treatment of IFIs like fungal meningitis are progressing quickly both in terms of new agents against these infections and new ways of delivering both old and new agents. In the recent development of nanomedicines, IONP have gained increasing attention for biomedical applications [32, 33]. These particulate systems are ideal for high level of accumulation in the target tissues or organ due to their host cell tropism, biophysical nature, and low toxicity [195]. IONP are one among the few materials injected into the body, which are easily incorporated into the body's natural metabolic pathways [181]. The active pharmaceutical ingredients (APIs) bind to the surface of the IONP, which then released at the target site with the application of external localized magnetic field gradient or through specific targeting. This approach offers a possibility of reduction in nonspecific toxicities by administering lower but more accurately targeted doses [33, 195]. Additionally these IONP will improve the magnetic contrast for MRI and thus useful as diagnostic/imaging agent as well. Rapid advances in the development of new drug delivery systems ensure that therapy will be available in the near future against IFIs.

This novel nanoparticle drug has both the ability to be more effective against infections but also imaged by existing MRI, so the particles can be tracked to the infection site in the brain or spinal cord. Often the site of fungal infection in the brain is not known and this product represents a significant advancement for diagnosing and monitoring the improvement of the infection. The National Institutes of Health (NIH)

have placed emphasis on developing nanoparticle medicines that can also be imaged for diagnosis and monitoring. My designs and techniques will most likely develop into potential products for another NIH priority to find antibiotics that work on drug resistant micro-organisms. As part of my research I will attempt to show that established antibiotics can be made into nanomedicines that will be more effective in killing drug resistant bacteria. This research has a high priority for the Centers of Disease Control (CDC) as many of our current antibiotics do not work to eliminate certain deadly bacteria [196].

As a proof of concept, I am attempting to develop a formulation to treat deadliest IFIs such as cryptococcal meningitis. For any drug delivery system it's a challenge to cross a very tight blood brain barrier to reach the site of infection for effective treatment. Furthermore, the development of novel nanotheranostic targeted agents to treat IFIs, like fungal meningitis, is important for imaging and to improve targeting to infections.

1.6.2. Specific aims

The overall objective of this dissertation is to design, develop, characterize and evaluate a targeted nanotheranostic drug delivery system to diagnose and treat life threatening IFIs such as cryptococcal meningitis. IFIs have emerged as major cause of morbidity and mortality, particularly in immunocompromised patients. Patients at risk include individuals undergoing hematopoietic stem cell transplantation, solid organ transplantation, or major surgery and patients with AIDS, cancer, immunosuppressive therapy [5]. Current therapies with the most effective antifungal therapy, that includes AMB in different formulations cause adverse effects such as nephrotoxicity, infusion related toxicity, pharmacokinetic variability, drug interactions, limited routes of administrations, thus, limiting its usage [8, 10]. This leads to a formulation with improved therapeutic efficacy with reduced adverse effects. A better way to improve this is by developing a targeted delivery system to the specific site of action, lowering the required dose and keeping AMB away from healthy cells. Additionally it will be advantageous to integrate the imaging property within in the delivery system to follow the path of nanoparticles upon intravenous (IV) administration to diagnose and monitor the severity of the infection.

Therefore, the studies proposed here are intended to develop a nano theranostic targeted drug delivery system for imaging and treatment purpose. To address these development processes, *in vitro* models of the fungal clinical isolates will be used to prove proof of concept. This contains four main objectives. First, formulate an IONP (35-50nm) containing AMB and coated with BSA (bovine serum albumin)/HSA (human serum albumin), applying a layer by layer approach. Then, convert the above formulated system in to a targeted drug delivery system by targeting with AMB itself in low quantity (AMB-IONP). Second, evaluate the uptake and efficacy of formulated targeted delivery system by performing *in vitro* studies in fungal clinical isolates. Third, evaluate the cellular uptake mechanisms and intracellular trafficking for formulated targeted delivery system by performing *in vitro* studies in selected fungal clinical isolates. Fourth,

lyophilize and develop a stable AMB loaded targeted IONP (AMB-IONP). It is hoped that these *in vitro* studies will produce a proof of concept that will allow pre-clinical animal studies. The information generated from these studies may potentially cure IFIs with better therapeutic efficacy and minimum toxicity, when compared to the current therapy.

The central hypothesis of my project was that the permeability and uptake of AMB-IONP in fungal clinical isolates is expected to improve the therapeutic efficacy of AMB while minimizing dose associated toxicities in the treatment of IFIs compared to AMB-D. Therefore, in order to test the hypothesis, the following specific aims were proposed:

- **Aim 1. Formulation development and characterization of a targeted drug delivery system of iron oxide nanoparticles encapsulated AMB (AMB-IONP).** The purpose of this specific aim is to design, formulate, prepare and characterize AMB-IONP. AMB-IONP will be prepared via layer by layer approach; initially either AMB or albumin will be conjugated to the oleic acid coated IONP, followed by adsorption of either albumin or drug, respectively. Alternate approaches are also used such as double layers of AMB and single layer of drug and albumin mixture to develop different formulation designs of the AMB-IONP. After preparation, AMB-IONP will be characterized for physicochemical properties (e.g. size, ζ -potential, AMB loading [with developed and validated high performance liquid chromatography (HPLC) assay], drug release, and drug release mechanisms). Several designs will be developed. Formulation and process parameters that influence size, ζ -potential, drug loading and drug release in the IONP will be identified and optimized to produce a targeted AMB-IONP formulation with a size range 35-50 nm for IV administration with sustain release profile.
- **Aim 2. In vitro evaluation of cellular uptake and efficacy of AMB-IONP in fungal clinical isolates.** Once the formulations are complete, *in vitro* studies are undertaken in target cells. The susceptibility testing for targeted AMB-IONP will be performed and compared against AMB-D (commercial product) on clinical isolates of *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, and *Candida tropicalis* using microbroth dilution assay [197]. The cellular uptake of AMB-IONP will be observed with a phase contrast microscope and will be determined quantitatively with flow cytometry. The uptake and efficacy of AMB-IONP and AMB-D will be compared to controls.
- **Aim 3. In vitro evaluation of cellular uptake mechanism and intracellular trafficking of AMB-IONP in fungal clinical isolates.** After the determination of cellular uptake and efficacy of AMB-IONP, *In vitro* cellular uptake mechanism and intracellular trafficking of the delivery system will be evaluated by using techniques including confocal microscopy, flow cytometry, and electron microscopy.
- **Aim 4. Development of stable lyophilized formulation of targeted AMB-IONP.** The purpose of this specific aim is to improve the shelf-life of the targeted AMB-IONP. AMB-IONP formulations will be prepared in the presence or

absence of lyoprotectants. Larger batches of lyophilized test formulations reduce the batch to batch variation of making daily small laboratory batches. The formulations will be subjected to lyophilization process to preserve AMB-IONP as dry powder. As a result of lyophilization, the original AMB-IONP formulation properties are expected to be retained with minimal or no damage after reconstitution. The stability of the reconstituted lyophilized product will be studied for up to six hours.

CHAPTER 2. DEVELOPMENT AND VALIDATION OF HPLC METHOD TO QUANTIFY AMPHOTERICIN B*

AMB, an amphoteric fermentation compound produced by the South American soil gram positive bacterium *Streptomyces nodosus* and is considered as a gold standard for treating severe systemic fungal infections [198]. Long-term usage of AMB leads to high incidence of adverse effects, predominantly nephrotoxicity and infusion-related toxicity. However, several formulations of AMB were developed to reduce the toxicity and to improve the therapeutic efficacy, they still cause considerable amount of toxicity [11, 199]. Hence, a careful monitoring of patients under treatment with AMB is required and demands for a simple, rapid, sensitive, and inexpensive method to determine AMB in biological samples like plasma and thereby determine its pharmacokinetic, bioequivalence, and toxicokinetic profile are needed.

Several analytical methods have been reported for the determination of AMB in biological samples like plasma using HPLC [200-213]. However, some of the reported HPLC methods have used salts in their mobile phase [204, 209, 210], which lowers the life-time of the column by significantly increasing the risk of saturation, breakdown or over pressure in the column. Longer retention times in a few reported methods [201, 205, 206, 212] require more time to analyze the samples and also consume more solvents. Though a reported method indicated short retention time for the elution of AMB, the peak resolution and symmetry is questionable [203].

The aim of this study was to develop and validate a simple, rapid, sensitive and reproducible isocratic HPLC method with a well resolved symmetrical peak of interest for the estimation of AMB in plasma samples. This method can be easily established in research and clinical laboratories for analyzing large number of plasma samples obtained for pharmacokinetic, bioequivalence or toxicokinetic studies after administration of AMB.

2.1. Materials and Methods

2.1.1. Reagents and chemicals

AMB USP analytical sample standard was obtained from MP Biomedicals (Santa Ana, CA, USA) and HPLC grade methanol, acetonitrile, dimethyl sulfoxide (DMSO), acetic acid and water were obtained from Fisher (Pittsburg, PA, USA). Drug-free human

* Adapted with permission. Balabathula, P., et al., *Rapid Quantitative Evaluation of Amphotericin B in Human Plasma, by Validated HPLC Method*. J Bioequiv Availab, 2013. 5: p. 121-124.

plasma was purchased from Innovative Research (Novi, MI, USA).

2.1.2. Instrumentation

All chromatographic studies were conducted on validated and qualified equipment. Chromatography was performed with a Waters HPLC Alliance system on an e2695 separations module with a Waters 2998 photo-diode array (PDA) detector (Milford, MA, USA). Samples were injected using a Waters auto injector and the instrument was controlled by use of Empower2® software (Milford, MA, USA). Components were separated on a Waters XBridge™ C18 reversed-phase column (Milford, MA, USA) with 150mm X 4.6 mm dimensions and 3.5 µm particle size. The column was kept thermostatic at 30°C in a Waters column oven (Milford, MA, USA).

2.1.3. Chromatographic conditions

The developed method used for detection and quantification of AMB was an isocratic method using an XBridge™ C18 reversed-phase column with a mobile phase of acetic acid (0.73%)-acetonitrile (59:41, v/v). The flow rate of the solvent was 1.0 mL/min with a runtime of 6 minutes. Samples were injected into the column at a constant volume of 20 µL and a PDA detector at 408 nm was employed to obtain the responses of varied concentrations of AMB. The column was maintained at 30°C.

2.1.4. Statistical methods

Standard calibration curves were plotted using peak areas and concentrations of the standard solutions. The data were fit to a linear model using a least squares regression analysis. The back calculated concentrations of the standards and the plasma samples (calculated using regression line) were used to determine the means, standard deviations (SD), and % coefficient of variation (%CV) at each concentration.

2.1.5. Preparation of standards for calibration curve

AMB stock solutions were prepared each day of analysis, by dissolving an accurate amount of USP standard AMB in the mixture of HPLC grade DMSO-methanol (50:50, v/v). The standard concentrations of 1000, 800, 600, 400, 200, 100, and 50 ng/mL were prepared by serial dilution in methanol. Each standard solution was injected (20 µL/injection) in sextuplicate and chromatographed under the described conditions. The method developed for the analysis of AMB was validated by developing a standard calibration curve with the standard concentrations on three different days. A different stock solution was prepared each day, respectively. The following parameters were calculated for the validation of the AMB assay; linearity, accuracy, intra-day and inter-day precision, limit of detection (LOD), limit of quantification (LOQ), and ruggedness.

2.1.6. Method for determining linearity and accuracy

The standard dilutions of AMB (1000- 50 ng/mL) were injected in series and the peak area responses were recorded. The standard concentrations were plotted against each peak area. A line of least squares regression was constructed, the slope and intercept were calculated and the coefficient of determination was determined for each calibration curve. Accuracy was determined by quantifying standard solutions in sextuplicate followed by the determination of the mean and % nominal.

2.1.7. Method for determining precision, LOD, and LOQ

Precision was calculated in accordance with International Conference on Harmonisation (ICH) guidelines for both repeatability and intermediate precision [214]. Repeatability of the method was determined as intra-day variation and intermediate precision of the assay was determined by inter-day variation [215, 216]. In order to determine both the intra-day and inter-day precision, AMB solutions at six different concentrations (1000, 800, 400, 200, 100, and 50 ng/mL) were determined in sextuplicate and mean and % CV were calculated. LOD and LOQ were determined by applying the **Equation 2-1** and **Equation 2-2** [217];

$$LOD = 3.3\sigma/m \quad (\text{Eq. 2-1})$$

$$LOQ = 10\sigma/m \quad (\text{Eq. 2-2})$$

where σ is the standard deviation of the intercept of regression line, and m is the slope of the calibration curve.

2.1.8. Ruggedness

Ruggedness of the method developed is evaluated by testing the reproducibility of the AMB peak resolution and its retention time obtained under a variety of normal test conditions, such as different laboratory, different analyst, different instruments, different lots of reagents, and different days.

2.1.9. Plasma extraction and preparation of the samples

The method of extraction was adapted from previously reported protocol by R. Lopez-Galera *et al* [203]. Human plasma samples (100 μ L) were spiked with AMB solution (20 μ L) to obtain the plasma concentrations of 5, 2, 1, and 0.5 μ g/mL and incubated at ambient temperature for 10 minutes. Spiked drug was extracted by adding 680 μ L of methanol to spiked plasma samples, vortex for 30 secs and sonicated for 3 minutes. The samples were then centrifuged at 20000 g for 5 min at 4°C and supernatant was assayed for recovery of AMB in triplicates by using validated HPLC assay. A

control sample of plasma was prepared in the similar way but without adding AMB.

2.2. Results and Discussion

2.2.1. HPLC method development

An HPLC method was developed to measure AMB concentrations from 1000 to 50 ng/mL in plasma samples by using XBridge™ C18 column and simple mobile phase. Different flow-rates and ratios of the mobile phase were studied in order to obtain a well resolved symmetrical AMB peak with a shorter retention time. The optimal separation was obtained with a mobile phase of acetic acid (0.73%)-acetonitrile (59:41, v/v) and with a flow-rate of 1 mL/min. The AMB peak in the standard solutions indicated a baseline separation with a retention time of approximately 2.7 minutes. The suitability of the method for analysis of AMB was confirmed by analysis of AMB concentration in each spiked plasma sample. Example chromatograms obtained for drug-free human plasma and AMB spiked human plasma are shown in **Figure 2-1**. No other peak is eluted with retention time similar to AMB in blank human plasma.

2.2.2. Linearity and accuracy

The linearity of the peak area versus the concentration (amount) was studied in the range of 1000-50 ng/mL for AMB and the results were subjected to statistical analysis using a linear-regression least-squares method. The linear regression data for the calibration curve, as shown in the **Table 2-1**, is indicative of a significant linear relationship between amount and peak area over the range studied. The calibration curves were found to be linear with a correlation coefficient (r^2) of 0.9964-0.9999. There were no significant inter-day differences between the slopes of the calibration curves. The low values of the standard deviation and standard error of slope indicates lack of deviation from the linearity or analytical bias in the calibration curve.

The accuracy of the method was determined by calculating the mean concentrations and % nominal mean for concentrations 1000 to 50 ng/mL for the three standard curves of AMB (**Table 2-2**). The % nominal mean (93.9-111.61%) indicate the method is acceptably accurate.

2.2.3. Precision, LOD and LOQ

Precision was determined, as both repeatability and intermediate precision, in accordance with ICH recommendations. Repeatability of the method was determined as intra-day variation, and intermediate precision was calculated by determining inter-day variation for sextuplicate determinations of AMB at six different concentrations (1000 and 50 ng/mL) on three different days for three calibration curves. Calculated values of

Figure 2-1. A representative chromatograms of drug-free plasma (a) and AMB spiked human plasma (b), retention time (t_R) = 2.7 minutes

Table 2-1. Linear regression data for calibration curve (n=3)

Parameter	Value
Linearity range (ng/mL)	50-1000
Regression equation	$y=129.33x - 1050.83$
Correlation coefficient (r^2) $\pm$ SD	0.9981 ± 0.0018
Slope $\pm$ SD	129.33 ± 4.04
Standard error of slope	2.33

Table 2-2. Accuracy and intra-day precision of the method for the determination of AMB (n=6)

Days	Actual concentration (ng/mL)	Mean concentration (ng/mL)	SD	Nominal (%)	CV (%)
1	50	52.385	1.48	104.77	2.82
2	50	46.949	1.16	93.90	2.48
3	50	60.806	0.85	111.61	1.39
1	100	101.626	0.98	101.63	0.96
2	100	90.363	1.50	99.36	1.66
3	100	109.939	1.32	109.94	1.20
1	200	196.742	0.85	98.37	0.43
2	200	198.633	1.30	99.32	0.66
3	200	192.351	1.97	96.18	1.02
1	400	399.164	1.47	99.79	0.37
2	400	408.131	1.83	102.03	0.45
3	400	385.919	2.42	96.48	0.63
1	800	796.896	2.07	99.61	0.26
2	800	838.937	2.82	104.87	0.34
3	800	776.791	3.69	97.10	0.47
1	1000	1003.187	5.57	100.32	0.56
2	1000	966.989	3.94	96.70	0.41
3	1000	1024.862	2.64	102.42	0.26

SD: Standard deviation, CV: Coefficient of Variance.

mean and %CV for determination of repeatability and intermediate precision are listed in **Table 2-2** and **Table 2-3**. The low values of %CV indicate the acceptable repeatability and intermediate precision of the method. The LOD and LOQ of AMB of the developed method were found to be 9.98 and 29.97 ng/mL, respectively. This sensitivity is achieved with just 20 μ L of injection volume and can be improved with higher injection volumes.

2.2.4. Ruggedness

Under a variety of normal test conditions as mentioned in the methods section, the AMB peak was found to be well resolved from all other peaks with the retention time of 2.7 minutes, illustrating the ruggedness of the method.

2.2.5. Recovery

Mean recovery of AMB from human plasma was found to be 88% at concentrations of 500 to 5000 ng/mL. Recoveries and the accuracy of the assay in determining AMB in spiked human plasma samples are summarized in **Table 2-4**. The deviation from theoretical values is under 15% at all concentrations levels studied.

2.3. Conclusions

A validated isocratic HPLC method has been developed for the determination of AMB in human plasma samples. The method is simple, rapid, accurate, and precise. Additionally, relatively shorter retention times (<3 min per injection) of AMB allow the analysis of a large number of samples in a short period of time. Therefore, this rapid and inexpensive method should be suitable for the routine analysis of AMB in dosage forms, clinical settings and to evaluate its pharmacokinetic, bioequivalence, and toxicokinetic profile.

Table 2-3. Inter-day variability of the method for the determination of AMB

Actual concentration (ng/mL)	Mean concentration (ng/mL) (<i>n</i>=18)	CV (%)
50	53.380	11.19
100	100.642	8.29
200	195.908	1.55
400	397.738	2.40
800	804.208	3.33
1000	998.124	2.47

CV: Coefficient of Variance.

Table 2-4. Accuracy and recovery of AMB in spiked human plasma (n=3)

Plasma concentration (µg/mL)	Spiked	Found	%Recovery (mean±SD)	CV (%)
5	600	535.380	89.23±1.88	0.27
2	240	204.528	85.22±2.37	0.90
1	120	105.132	87.61±0.52	0.39
0.5	60	54.768	91.28±1.47	1.90

SD: Standard deviation, CV: Coefficient of Variance.

CHAPTER 3. DESIGN, DEVELOPMENT AND CHARACTERIZATION OF AMPHOTERICIN B LOADED IRON OXIDE NANOPARTICLES

IFIs are considered life threatening and are associated with high rate of morbidity and mortality, most commonly among immunocompromised patients. IFIs account for more than 60 percent of the mortality rates for some of these infections in certain situations, and remains one of the greatest challenges for clinicians who care for immunocompromised patients [8]. Patients at high risk for IFIs comprise those undergoing treatment with cancer chemotherapy, HIV infected patients, and acute leukemia or hematopoietic stem cell transplant receivers [10]. With improvements in medical care that extend survival of critically ill patients (e.g. HIV), the incidence of IFIs was further increased with aggressive treatment strategies [10]. Consequently, this resulted in a greater number of immunocompromised patients. Additionally, among these hosts an increasing variety of fungal species has been found [5, 193, 194]. Annually, about one million new cases reported worldwide for the HIV infected patients diagnosed with cryptococcal meningitis [2]. Out of these one million, more than 62 percent deaths have been estimated.

AMB is one of the most effective drugs used in chemotherapy of IFIs. It is a natural product isolated from a filamentous bacterium *Streptomyces nodosus* [8]. AMB was discovered in 1955 and later introduced into the market by approval of FDA in 1958 as AMB-D [218]. AMB-D is still considered a gold standard for treatment of many deadly fungal infections [8, 21]. AMB has widest spectrum of activity against fungal pathogens including *Candida* species, *Cryptococcus neoformans*, and *Coccidioides immitis* [9]. For this reason AMB was chosen as the model drug. AMB-D is a parenteral drug product, with a high rate of toxic effects including infusion-associated adverse effects and dose-associated nephrotoxicity [8, 9, 219]. Unfortunately this limits treatment options with AMB-D. So far there is no ideal antifungal agent for treatment of patients with progressive and potentially life-threatening fungal infections including IFIs. Older agents are toxic and newer agents including azoles and echinocandins are associated with aberrant pharmacokinetic profiles, drug interactions, a narrow spectrum of activity and limited routes of administration, thus limiting their use [8]. Three lipid formulations of AMB have been developed to address these issues, but the relative efficacy and safety of these drugs has not been adequately studied and published [8, 10]. The antifungal effect of these AMB lipid agents on fungal cells is highly beneficial for the therapy, but adverse effects are still present mostly due to their effect on normal tissues. Regardless of numerous innovations in antifungal therapy, there is still a demand for better therapy of IFIs (e.g. fungal CNS infections and pneumonias). Thus, there is a need for an effective drug delivery system that produces antibiotic concentrations of the drug at the fungal sites without affecting normal tissues.

An ideal drug delivery system for fungal infection treatment would be the one that has ability to target and to kill the fungal cells while affecting as few healthy cells as possible. The developments in treatment options are progressing rapidly both in terms of novel agents and ways of delivering both old and new agents against these infections.

With the recent improvement of nanomedicines, magnetic nanoparticles have gained increasing attention for its biomedical applications [32]. There are several advantages with these particulate systems including a high level of accumulation in the target tissues or organs due to their host cell tropism, biophysical nature, and low toxicity [195]. Upon administration into the body, IONP are easily incorporated into the body's natural metabolic pathways [181]. In addition, flexibility in the design of IONP allows tailoring to achieve layering/attaching different components like BSA and AMB. Layering allows sequential release of each layer as nanoparticles encounter various parts of the body. First, with this approach both passive as well as active targeting ability is achievable. The drug binds to the surface of the IONP and then released at the target site with the use of external localized magnetic field gradient or through specific targeting. With this approach there is a possibility of minimizing nonspecific toxicities by administering smaller but more accurately targeted doses [33, 195]. Secondly, IONP can be used as diagnostic/imaging agent due their capability of improving the magnetic contrast for MRI.

The physicochemical characteristics of these colloidal magnetic nanoparticles are critical for creating therapeutic advantages including both a prolonged blood circulation time as well as increased bioavailability of these particles at the infection site [32, 220]. First, the magnetic properties and internalization of IONP are potentially driven by the small size and the concentration of the nanoparticles [221]. Upon systemic administration the larger particles with diameters more than 200 nm are typically sequestered by the spleen and RES as a consequence of mechanical filtration. These particles are then eventually cleared by the cells of the phagocyte system. In contrast, the extremely small particles of less than 10 nm in diameter in size will be rapidly cleared through extravasations and renal clearance. In nanomedicines the optimum particle size range is often from 10 to 100 nm for parenteral administration. This size range evades much of the RES of the body and still penetrates the very small capillaries to have an effective distribution in targeted tissues [32, 222]. The RES of body, predominantly Kupffer cells in the liver engulf these IONP due to the hydrophobic surface. Thus, surface coating of IONP with amphiphilic substance like poly ethylene glycol or albumin provide a shielding effect and prevent recognition and binding of opsonins. Hence, these IONP can escape RES, significantly improving the blood circulation time to offer a longer time for particles to reach the site of infection through improved trapping of the particles [23, 223].

Secondly, apart from size, the particle size distribution is an essential characteristic of nanoparticle products. Particle size distribution is measured by a parameter known as polydispersity index (PDI). Theoretically, PDI is the measure of the width of particle size distribution and expressed by a dimensionless quantity ranging from 0 to 1 [224]. The size distribution is considered monodispersed if the PDI value is ≤ 0.1 [225]. For the present study, PDI of ≤ 0.4 is quite small and was considered acceptable. Thirdly, surface charge of the nanoparticles is also an important parameter and is measured by ζ -potential. The electrostatic potential at the electrical double layer surrounding the nanoparticles in solution is identified as ζ -potential. The ζ -potential of $\sim \pm 10$ mV is considered neutral whereas ζ -potential of $> \pm 30$ mV considered strongly

electropositive or electronegative [226]. The particles with near neutral ζ -potential show a tendency towards faster aggregation leading to the possibility of enhanced clearance by the RES. A small surface charge offers better colloidal stability and less aggregation of nanoparticles and leading to a longer shelf life upon storage [227]. Positively charged nanoparticles were found to interact more with the negatively charged cell membranes thereby enhancing toxicity to healthy tissues. On the other hand, negatively charged nanoparticles were found to be safe and non-toxic to healthy tissues [228]. IONP with partially negative ζ -potential are desirable for both *in vivo* stability upon intravenous administration and for colloidal stability upon storage.

An ideal carrier for therapeutic targeting to IFIs must have a longer blood circulation time and should possess targeting capability to increase total dose going to the infection site. *In vivo* circulation time can be increased by keeping the size of IONP formulation under 100 nm and/or modifying the surface with albumin. Targeting capability can be achieved by attaching specific ligands onto the surface of the IONP. Ergosterol is highly expressed in the lipid bilayer of fungal cell membranes [16, 18, 21]. AMB has a strong binding affinity towards ergosterol [16] and could be potentially used as a targeting agent as well. By having AMB bound to albumin on the IONP, they can be targeted to IFIs. Therefore, the objective of this work was to design, development and characterize of AMB-IONP formulations that serves as a targeted delivery system to selected fungi that can produce IFIs.

3.1. Materials and Methods

3.1.1. Materials

The oleic acid coated IONP, activation buffer (50 mM sodium borate buffer, pH 5.5), coupling buffer (50 mM sodium borate buffer, pH 8.5), quenching buffer (1 M 2-(2-amino ethoxy) ethanol, pH 9.5), wash/storage buffer (borate buffer, pH 7.4), and SuperMag Separator™ magnetic separator were obtained from Ocean NanoTech (Springdale, AR, USA). EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) was purchased from Acros (Pittsburg, PA, USA) and NHS (sulfo-N-hydroxysuccinimide) was purchased from Thermo Scientific (Rockford, IL, USA). BSA (Cohn fraction V, lyophilized powder, MW~66000, purity 95-99%) and glutaraldehyde solution (25 % in water) were purchased from Sigma-Aldrich (St. Louis, MO, USA). AMB USP analytical standard was obtained from MP Biomedicals (Santa Ana, CA, USA) and HPLC grade methanol, acetonitrile, DMSO, acetic acid, phosphate buffer saline (PBS), and water were obtained from Fisher (Pittsburg, PA, USA). Tris-acetate-EDTA (TAE) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Molecular biology grade agarose, standard, low electroendosmosis was purchased from VWR International (Randor, PA, USA). Fetal bovine serum (FBS) was purchased from ATCC (Manassas, VA, USA).

3.1.2. Development of formulation designs

Different designs of AMB-IONP were prepared using a layer-by-layer approach. The protein conjugation protocol used for the development of AMB-IONP formulation designs was adapted from Ocean NanoTech protocol [229].

3.1.2.1. Design A. In design A, the order of the layers from inside to outside are IONP, BSA, and AMB (**Figure 3-1A**). The oleic acid coated IONP (1 mg) were initially treated with 100 μ L of activation buffer and then immediately the surface groups on IONP were activated with 100 μ L of 2 mg/mL EDAC and 1 mg/mL NHS. The mixture was reacted for 10 minutes with continuous mixing at ambient temperature. Then 400 μ L of the coupling buffer was added to the activated IONP and mixed well. It was immediately followed by the adding of at least 1 mg of BSA dissolved in a maximum volume of 500 μ L to the activated IONP mixture and mixed well. The mixture was reacted for two hours with continuous mixing at ambient temperature. Then 10 μ L of the quenching buffer was added, mixed well and incubated at ambient temperature for at least 10 minutes. The reaction mixture was transferred into a plastic cuvette, 3 mL of wash/storage buffer added, and mixed well gently with a pipette. The cuvette was placed into the magnetic separator and allowed the IONP to separate at 4°C for 10-12 hours. Without touching the inner walls of the cuvette, the liquid was carefully removed with a Pasteur pipette. In this way most of the free material not attached to the IONP can be removed. The cuvette was removed and the IONP resuspended in 3 mL of wash/storage buffer with gentle mixing with a pipette (second washing). Then subjected to magnetic separation of the nanoparticles for another 10-12 hours. The liquid was carefully aspirated and the BSA coated IONP suspended in 0.5 mL AMB solution in DMSO (50-100 μ g/mL). The mixture was incubated at ambient temperature for about 2 hours for adsorption/binding of the drug to albumin to obtain AMB-IONP of design A. Three mL of wash/storage buffer was added and mixed gently with a pipette and transferred into a plastic cuvette for magnetic separation for 10-12 hours, followed by carefully aspiration of the liquid with a Pasteur pipette and resuspension in 3 mL of wash/storage buffer for additional washing. The mixture was then subjected to the magnetic separation for 10-12 hours at 4°C. Finally, the liquid was aspirated and the nanoparticles resuspended in 400-500 μ L of wash/storage buffer and stored at 4°C until further usage.

3.1.2.2. Design B. In design B, the order of the layers from inside to outside are IONP, AMB, and BSA (**Figure 3-1B**). The oleic acid coated IONP (1 mg) were initially treated with 100 μ L of activation buffer and then immediately the surface groups on IONP were activated with addition of 100 μ L of 2 mg/mL EDAC and 1 mg/mL NHS. The mixture was reacted for 10 minutes with continuous mixing at ambient temperature. Then added 400 μ L of the coupling buffer to the activated IONP, mixed well and immediately added 0.5 mL AMB solution in DMSO (50-100 μ g/mL). The mixture was then reacted for two hours with continuous mixing at ambient temperature. Then 10 μ L of the quenching buffer was added, mixed well and incubated at ambient temperature for at least 10 minutes. Then the nanoparticles were washed twice with 3 mL of wash/storage

Figure 3-1. Formulation designs of AMB-IONP

The above figure represents formulation designs of AMB-IONP. IONP represents oleic acid coated IONP with core size of 15 nm. The formulation designs A, B, C, D, and E are represented in the figure.

buffer each time and subjected for magnetic separation for 10-12 hours at 4°C, followed by carefully aspiration of the liquid. Next, suspended the AMB coated IONP in 0.5 mL BSA solution (2-3mg/mL) in coupling buffer. The mixture was incubated at ambient temperature for about 2 hours for binding of albumin to drug coated IONP in order to obtain AMB-IONP of design B. Then the nanoparticles were washed twice with 3 mL of wash/storage buffer each time and subjected for magnetic separation for 10-12 hours at 4°C. Finally, the liquid was aspirated and the nanoparticles were resuspended in 400-500 μ L of wash/storage buffer and stored at 4°C until the usage.

3.1.2.3. Design C. In design C, the order of the layers from inside to outside are IONP, AMB, BSA, and AMB (**Figure 3-1C**). The design C is the extension of the design B. So, after the preparation of design B, the nanoparticles are suspended in 0.5 mL AMB solution in DMSO (50-100 μ g/mL) and incubated at ambient temperature for about two hours for binding for AMB to albumin on nanoparticles in order to obtain AMB-IONP of design B. Then the nanoparticles were washed twice with 3 mL of wash/storage buffer each time and subjected for magnetic separation for 10-12 hours at 4°C. Finally, the liquid was aspirated and the nanoparticles were resuspended in 400-500 μ L of wash/storage buffer and stored at 4°C until the usage.

3.1.2.4. Design D. In design D, the order of the layers from inside to outside are IONP, AMB bound BSA (**Figure 3-1D**). To ~0.5 mL of BSA solution (2-3mg/mL) in coupling buffer added 40 μ L of AMB (5 μ g/mL), mixed gently and incubated for 1-2 hours at ambient temperature to obtain drug bound albumin solution. The oleic acid coated IONP (1 mg) were initially treated with 100 μ L of activation buffer and then immediately the surface groups on IONP were activated with 100 μ L of 2 mg/mL EDAC and 1 mg/mL NHS. The mixture was reacted for 10 minutes with continuous mixing at ambient temperature. Then 400 μ L of the coupling buffer was added to the activated IONP, mixed well and immediately followed by adding of 0.5 mL of AMB bound BSA mixture. The mixture was reacted for two hours with continuous mixing at ambient temperature. Then 10 μ L of the quenching buffer was added, mixed well and incubated at ambient temperature for at least 10 minutes. Then the nanoparticles were washed twice with 3 mL of wash/storage buffer each time and subjected for magnetic separation for 10-12 hours at 4°C. Finally, the liquid was aspirated and the nanoparticles were resuspended in 400-500 μ L of wash/storage buffer and stored at 4°C until the usage.

3.1.2.5. Design E. In design E, the order of the layers from inside to outside are IONP and AMB (**Figure 3-1E**). The oleic acid coated IONP (1 mg) were initially treated with 100 μ L of activation buffer and then immediately the surface groups on IONP were activated with 100 μ L of 2 mg/mL EDAC and 1 mg/mL NHS. The mixture was reacted for 10 minutes with continuous mixing at ambient temperature. Then 400 μ L of the coupling buffer was added to the activated IONP, mixed well and immediately added 0.5 mL of AMB solution (50-100 μ g/mL). The mixture was reacted for two hours with continuous mixing at ambient temperature. Then 10 μ L of the quenching buffer was

added, mixed well and incubated at ambient temperature for at least 10 minutes. Then the nanoparticles were washed twice with 3 mL of wash/storage buffer each time and subjected for magnetic separation for 10-12 hours at 4°C. Finally, the liquid was aspirated and the nanoparticles resuspended in 400-500 µL of wash/storage buffer and stored at 4°C until the usage.

3.1.3. Success of conjugation

The success of conjugation of BSA to IONP was monitored with agarose gel electrophoresis by running the all formulation designs alongside controls, IONP without drug and unlabeled IONP with BSA after 30 minutes of reaction. The agarose gel electrophoresis protocol was adapted from Ocean NanoTec [230]. Briefly, enough 1x Tris-Acetate was diluted from 10x for a 40 mL gel and to completely fill the gel chamber and set aside. One and one-half percentage (1-1.5%) agarose gel was prepared by weighing out approximately 0.4-0.6 g of the agarose powder and then transferred into a 100 mL Erlenmeyer flask. Then 40 mL of 1x TAE buffer was transferred into the flask containing agarose powder and swirl to ensure homogeneous distribution of the agarose powder in the TAE buffer. The agarose-TAE buffer mixture was heated until it begins to boil. The flask was swirled very well to allow all the agarose dissolve in the buffer. The mixture was continued heating until the agarose was completely dissolved in the buffer without the mixture boiling over. The mixture was then set aside to cool down just enough to ensure the solution will not melt the gel tray. While the gel solution was cooling down the gel chamber was filled with enough 1x TAE buffer until the chamber was filled half way. Once the agarose buffer was cooled enough to hold in hand without burning feeling, the solution was transferred into the gel tray with the well comb fixed. After the gel was then allowed to cool completely and solidified the combs are removed. The tray was inserted properly into the gel chamber and enough 1x TAE buffer was poured into the chamber to cover the gel and fill the wells. The samples of 5 µL of IONP and formulation designs were pipetted into the wells of the gel. The gel was allowed to run at 100V for 30 minutes.

3.1.4. Estimation of drug loading

Loaded AMB amount in the AMB-IONP was determined by a developed and validated HPLC method described in Chapter 2 [198]. All chromatographic studies were conducted on validated and qualified equipment. The HPLC system consists of a Waters HPLC Alliance system on an e2695 separations module with a Waters 2998 PDA detector (Milford, MA, USA). Samples were injected using a Waters auto injector and the instrument was controlled by use of Empower2® software (Milford, MA, USA). Components were separated on a Waters XBridge™ C18 reversed-phase column (Milford, MA, USA) with 150 mm X 4.6 mm dimensions and 3.5 µm particle size. The column was kept thermostatic at 30°C in a Waters column oven (Milford, MA, USA). The developed method used for detection and quantification of AMB was an isocratic method with a mobile phase of acetic acid (0.73%)-acetonitrile (59:41, v/v). The flow rate of the

solvent was 1.0 mL/min with a runtime of 6 minutes. The drug was extracted from the formulation by dispersing the AMB-IONP in DMSO at a dilution factor of 25 and then subjecting to sonication for 30 minutes. The dispersion was then subjected to magnetic separation for 10-12 hours at 4°C. An aliquot was taken for HPLC analysis. Samples were injected into the column at a constant volume of 20 μ L and a PDA detector at 408 nm was employed to obtain the responses of AMB.

3.1.5. Determination of particle size and zeta (ζ) potential

The mean hydrodynamic size (Z-average) of IONP and AMB-IONP were determined by dynamic light scattering (also known as photon correlation spectroscopy) technique using Malvern Zetasizer Nano ZS90 (Westborough, MA, USA) 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-average for size. Each sample was determined in duplicate. ζ -potential was estimated with electrophoretic light scattering (also known as laser doppler micro-electrophoresis) technique with the Malvern Zetasizer Nano ZS90. Samples were suitably diluted in HPLC grade water filled into the disposable capillary cell (DTS1070) for analysis.

3.1.6. Morphological characterization by TEM

Morphology of the IONP and design D of AMB-IONP were characterized by transmission electron microscopy (TEM) using a negative staining technique. Sequential two droplet method was used for staining samples with uranyl acetate on 400-mesh formvar support film on copper specimen grid from Electron Microscopy Sciences (Hartfield, PA, USA) and air dried. The TEM images were obtained at various magnifications using JEOL 2000EX transmission electron microscope (Peabody, MA, USA) equipped with high resolution digital camera. The IONP cores and formulation design C and D were imaged with TEM.

3.1.7. Drug release study design

3.1.7.1. Drug release in 1x PBS. Drug release was studied in dissolution media containing 1x PBS of pH 7.4 using previously reported dialysis method [231]. The in-house set up for *in vitro* drug release experiment was shown in **Figure 3-2**. A 100 μ L portion of the design A, B, and C of AMB-IONP dispersion was pipetted into each Slide-A-Lyzer™ MINI dialysis microtubes with a molecular weight cutoff of 3500 Da (Pierce, Rockford, IL, USA). Minidialysis tubes were aligned in a floater which was placed in a beaker containing 1000 mL of the dissolution medium (PBS pH 7.4) above magnetic stirrer stirring at 75 rpm. The whole set up was arranged inside an oven maintained at 37°C. At predefined time point, minidialysis tubes containing samples were taken out,

Figure 3-2. Schematic diagram of the in-house experimental setup for *in vitro* drug release study

The AMB-IONP liquid dispersion was transferred into minidialysis tubes (MWCO 3.5 k Da). The tubes were inserted into 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.

extracted in DMSO with sonication for 30 minutes at ambient temperature. After magnetic separation for 10-12 hours at 4°C, the samples were injected in HPLC to quantify the amount of drug released in specified time points. Data of the AMB release from formulation designs A, B, and C of AMB-IONP were analyzed for release kinetics by zero-order, first order, Higuchi, Hixon-Crowell, and Korsmeyer-Peppas models [232, 233].

3.1.7.2. Drug release in 1x PBS with 4% BSA. For this study design D was chosen as it was found to be a lead formulation, as described in Chapter 4. In order to further control the drug release the BSA on design D was cross-linked with glutaraldehyde (0.47% of BSA). Addition of a chemical cross-linker such as glutaraldehyde provides better stability, shape, insolubility at high temperatures, and reduced swelling. The glutaraldehyde was added to the AMB-IONP dispersion and mixed for 12 hours at ambient temperature. Then the particles were washed twice with wash/storage buffer and subjected to magnetic separation for 10-12 hours at 4°C. The liquid was aspirated out and the particles were dispersed in wash/storage buffer and stored at 4°C until further use. Drug release was studied for both with and without cross-linking agent in dissolution media containing 4% BSA in 1x PBS of pH 7.4 using dialysis method. A 100µL of sample was pipetted into each Slide-A-Lyzer™ MINI dialysis microtubes with a molecular weight cutoff of 3500 Da. Minidialysis tubes were aligned in a floater which was placed in a beaker containing 1000 mL of the dissolution medium above magnetic stirrer stirring at 75 rpm. The whole set up was prepared inside an oven maintained at 37°C. At predefined time point, minidialysis tubes containing samples were taken out, extracted in DMSO with sonication for 30 minutes at ambient temperature. After magnetic separation for 10-12 hours at 4°C, the samples were injected in HPLC to quantify the amount of drug released in specified time points. Data of the AMB release from formulation designs D of AMB-IONP with and without cross-linking were analyzed for release kinetics by zero-order, first order, Higuchi, Hixon-Crowell, and Korsmeyer-Peppas model.

3.1.8. Colloidal stability in FBS

The colloidal stability of design D of AMB-IONP in serum was performed according to the previously reported method [234, 235]. The AMB-IONP were suspended in 50% FBS with a final nanoparticle concentration of 1 mg/mL. The particles were concentrated to 2 mg/mL and an equal volume of FBS was then added. Samples were incubated at 37°C in a shaker with light shaking. At predetermined time points the samples were subjected to UV-Visible Spectrophotometer analysis. Due to interference of high concentration of plasma proteins at 50% FBS, absorbance measurements were conducted at 560 nm for each time point. The measurements were performed in triplicate at room temperature.

3.2. Results and Discussion

3.2.1. Determination of conjugation efficiency

The conjugation efficiency of BSA to the oleic acid coated IONP was qualitatively determined by running agarose gel electrophoresis. The unconjugated IONP were used as control, which in general will migrate faster than the BSA conjugated IONP [230]. It is also important to know that theoretically, the negatively charged nanoparticles will migrate towards the positive electrode and negatively charged nanoparticles will migrate towards the negative electrode. The results are shown in **Figure 3-3**. The control formulation without drug and formulation designs A, B, C, and D were migrated slower than the unconjugated formulation design E and IONP. These results confirm the successful conjugation of BSA onto the oleic acid coated IONP.

3.2.2. Determination of drug loading

There was no set target concentration of drug for AMB-IONP formulations. It only requires enough drug loaded into the IONP system to perform the job of eradicating fungal infection. A representative chromatogram of the AMB is shown in **Figure 3-4**. The AMB peak is well resolved from the adjacent peaks with retention time of 2.7 minutes. The drug loaded into the formulations was directly determined from each formulation by extracting the drug into DMSO. The drug loading was determined by the **Equation 3-1**.

$$\text{Drug loading} = \frac{\text{Amount of drug in nanoparticles } (\mu\text{g})}{\text{Amount of nanopartilces } (\text{mg})} \quad (\text{Eq. 3-1})$$

The amount of drug loaded in the formulations was calculated from standard curve which was linear between 0.050 to 1 $\mu\text{g/mL}$ with a coefficient of determination (r^2) value of 0.9987.

The effect of layer-by-layer approach on the formulation designs in terms of drug loading is shown in **Figure 3-5**. As expected, the difference in drug loading was observed with different formulation designs. In the first formulation design A the drug loading was found to be minimum with 2.7 ± 0.9 μg of AMB/mg of nanoparticle. The design A consists of albumin conjugating to the nanoparticle cores and then the drug is adsorbed onto it. Whereas the design B consists of attachment of AMB to the cores and then albumin is adsorbed onto it. With this approach the drug loading was slightly increased to 3.2 ± 2.0 μg of AMB/mg of nanoparticle. In order to increase the drug loading another layer of AMB was adsorbed onto the design B, resulting in design C. As expected the drug loading was increased to 8.2 ± 3.3 μg of AMB/mg of nanoparticle. So, the double layer of AMB increased the drug loading. In order to further increase the drug loading and to keep the formulation steps minimum design D was formulated, which consists of single coating of AMB/BSA mixture. As expected the drug loading was further increased

Figure 3-3. Gel electrophoresis of IONP and formulation designs Control, A, B, C, D, and E

The BSA unconjugated IONP and design E migrated faster than BSA conjugated formulations such as control and designs A, B, C, and D. This difference in migration confirms the success of conjugation of albumin onto BSA conjugated formulations. Since the BSA unconjugated particles are not coated with high molecular weight albumin, they migrated faster than BSA conjugated particles.

Figure 3-4. Representative HPLC chromatogram of AMB in formulation designs

The AMB peak was eluted at a reproducible 2.7 minutes retention time. The peak found to be symmetrical and well resolved from adjacent peaks. AU represents absorption units measured by UV spectroscopy.

Figure 3-5. Drug loading of AMB in all formulation designs

The highest AMB loading was observed with formulation design D. Mean values are shown on each histogram column. The data were presented as mean $\pm$ SD ($n \geq 3$).

to 13.6 ± 6.9 μg of AMB/mg of nanoparticle. The design E was developed as a control to other designs without attaching BSA and the drug loading was found to be 7.8 ± 2.5 μg of AMB/mg of nanoparticle. This study concluded that the design D has maximum drug loading to be a lead formulation.

3.2.3. Characteristics of AMB-IONP

The different formulation designs were prepared by a layer-by-layer approach. The mean hydrodynamic size of the cores i.e. oleic acid coated IONP were found to be 29.3 ± 1.0 nm (**Figure 3-6**) and in good agreement with manufacture specifications. After addition of additional layers onto these cores the mean size increased up to 35 nm. The mean hydrodynamic size of the formulation designs A, B, C, D, and E found to be 35.3 ± 2.5 , 35.9 ± 2.5 , 34.5 ± 2.3 , 34.3 ± 1.7 , and 30.1 ± 0.1 , respectively (**Figure 3-6**). There was no significant difference in mean size among the different formulation designs and the size about 35 nm is considered optimum and extremely small in nanomedicine which is favorable for longer circulation time and greater tissue penetration. These particles can escape RES, and also benefit from passive targeting due to EPR effect. The PDI is the measure of the polydispersity and it was found to be ≤ 0.2 , which was considered optimum (**Figure 3-7**).

The ζ -potential of both IONP cores found to be -42.3 ± 6.9 mV (**Figure 3-8**) and in good agreement with manufacture specifications. After addition of additional layers BSA and/or AMB onto these cores the ζ -potential increased up to -27 mV. The ζ -potential of the formulation designs A, B, C, D, and E found to be $\sim -27.5 \pm 8.0$, -26.6 ± 7.1 , -28.6 ± 8.1 , -31.1 ± 6.7 , and -35.8 ± 5.3 , respectively (**Figure 3-8**) indicating that these nanoparticles will have good colloidal stability. The ζ -potential measurements before and after coating of BSA and/or AMB showed an increase from ~ -42 mV to ~ -27 mV for all the formulation designs. This phenomenon could be attributed to the fact that both albumin and AMB positively charged and thus add some amount of positive charge to have net increase ζ -potential of ~ -27 mV.

The morphological characterization of both core nanoparticles and formulation designs were imaged by TEM with a negative staining technique. The TEM image of IONP cores is presented in **Figure 3-9**, which was imaged at 500,000X magnification. The image exhibited monodispersity of the nanoparticles that are spherical in shape. The size in diameter of these cores measured by TEM are ~ 15 nm, which are in good agreement with the manufacture specifications. The hydrodynamic size of oleic acid coated IONP is between 24 and 30 nm, but the IONP cores are about 15 nm. In the TEM images only the cores are denser and so clearly visible over the additional layers on them. Not all the nanoparticles are in focus due to the multiple layers of sample. The TEM images of formulation design C and D are exhibited in **Figure 3-10** and **Figure 3-11**, respectively. These images confirm that the design C and D formulations are monodispersed with spherical shape of nanoparticles. Even though the nanoparticle size that observed in TEM was in good agreement with that determined by dynamic light scattering (DLS) technique, there was slight reduction in size when measure through

Figure 3-6. Mean particle size of IONP and formulation designs

The mean particle size of IONP cores were between 25 and 30 nm and after attaching layers of BSA and/ AMB the size remained under 40 nm, which is considered optimum for nanomedicine. Mean values are shown on each histogram column. The data were presented as mean $\pm$ SD ($n \geq 3$). NP: nanoparticle.

Figure 3-7. PDI of IONP and formulation designs

The mean PDI of IONP cores and formulation designs were under 0.2, which is considered optimum for the project. Mean values are shown on each histogram column. The data were presented as mean $\pm$ SD ($n \geq 3$).

Figure 3-8. ζ -potential of IONP and formulation designs

The mean particle size of IONP cores were between 25 and 30 nm and after attaching layers of BSA and/ AMB the size remained under 40 nm, which is considered optimum for nanomedicine. Mean values are shown on each histogram column. The data were presented as mean $\pm$ SD ($n \geq 3$).

Figure 3-9. TEM image of IONP cores at 500,000X magnification

TEM image revealed spherical and monodispersity of the IONP cores and in good agreement with manufacture specifications.

Figure 3-10. TEM image of design C formulation of AMB-IONP at 500,000X magnification

TEM image revealed spherical and monodispersity of the AMB-IONP design C and in good agreement with DLS data.

Figure 3-11. TEM image of design D formulation of AMB-IONP at 500,000X magnification

TEM image revealed spherical and monodispersity of the AMB-IONP design D and in good agreement with DLS data.

TEM compared to DLS. This could be attributed to the fact that the 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 [236].

3.2.4. Drug release profile

In vitro dissolution study to obtain the drug release profile is considered as an essential part in drug development process [237]. There are several model dependent methods reported in literature, which are based on different mathematical functions that describe the dissolution profile [238, 239]. The most common applied mathematics models for drug delivery systems include zero order, first order, Higuchi, and Hixson-Crowell. After obtaining the dissolution curves for the drug release study of the formulation designs, the best fit is selected based upon the highest regression coefficient (r^2) value among the models. Korsmeyer-Peppas models was used to determine the drug release mechanism from the value of release exponent (n), which is slope of the regression equation of the dissolution curve [240].

3.2.4.1. Design A. The formulation design A consists of albumin attached to the IONP cores and then the drug AMB was bound onto it. The drug release profile was exhibited in **Figure 3-12**, which indicates a burst release of ~60% of cumulative drug release within first 3 hours, followed by a sustain release profile up to 72 hours. Upon study of the release kinetics the data indicated that it followed first order release kinetics best with r^2 of 0.8878 (**Figure 3-13**), which concludes that the drug released via diffusion controlled mechanism and the drug release rate was dependent on the concentration of drug in design A formulation of AMB-IONP. For the release mechanism study with Korsmeyer-Peppas release model exhibited high correlation for drug release from design A formulation at pH 7.4 (**Figure 3-14**). The diffusion exponent, n was about 0.18, which indicates the Fickian diffusion was the controlling factor of drug release mechanism. The initial burst release could be the effect of the diffusion of the drug from surface of the albumin coated onto the IONP.

3.2.4.2. Design B. The formulation design B consists of AMB attached to the IONP cores and then the albumin was bound onto it. The drug release profile was exhibited in **Figure 3-15**, which indicates a burst release of ~26% of cumulative drug release within first 3 hours, followed by a sustain release profile up to 72 hours. Upon study of the release kinetics the data indicated that it followed first order release kinetics best with r^2 of 0.7827 (**Figure 3-16**), which concludes that the drug released via diffusion controlled mechanism and the drug release rate was dependent on the concentration of drug in design B formulation of AMB-IONP. For the release mechanism study with Korsmeyer-Peppas release model exhibited good correlation for drug release from design B formulation at pH 7.4 (**Figure 3-17**). The diffusion exponent, n was about 0.64, which indicates the anomalous transport, non-Fickian diffusion was the controlling factor of

Figure 3-12. Cumulative % drug release from design A

The data were presented as mean $\pm$ SD (n=4). The study was conducted in release media of 1x PBS at 37°C. Sustained release of drug from AMB-IONP formulation design A was observed for about 72 hours. Initial burst release was observed up to 3 hours.

Figure 3-13. Drug release model fit for design A

The best fit for drug release kinetic model was found to be first order with r^2 of 0.8878. This concludes that the drug released via diffusion controlled mechanism and the drug release rate was dependent on the concentration of drug in design A formulation of AMB-IONP. Through diffusion drug slowly released from the surface.

Figure 3-14. Korsmeyer-Peppas model fit determination of drug release mechanism from design A

The diffusion exponent, n was found to be 0.18 indicating Fickian diffusion as controlling factor of drug release mechanism. Here, the drug is on surface and can easily diffuse into the medium. Fickian diffusion is dependent on concentration gradient and surface area of the particles.

Figure 3-15. Cumulative % drug release from design B

The data were presented as mean $\pm$ SD (n=4). The study was conducted in release media of 1x PBS at 37°C. Sustained release of drug from AMB-IONP formulation design B was observed for about 72 hours. Initial burst release was observed up to 3 hours.

Figure 3-16. Drug release model fit for design B

The best fit for drug release kinetic model was found to be first order with r^2 of 0.7827. This concludes that the drug released via diffusion controlled mechanism and the drug release rate was dependent on the concentration of drug in design B formulation of AMB-IONP. Through diffusion drug slowly released from the surface.

Figure 3-17. Korsmeyer-Peppas model fit determination of drug release mechanism from design B

The diffusion exponent, n was found to be 0.64 indicating anomalous transport, non-Fickian diffusion as controlling factor of drug release mechanism. This could be resulted from the internal stress applied by one part of the medium on another as diffusion takes place. The AMB layer is inside and bind to the IONP cores make it not diffuse and is concentration independent. This phenomenon may play a crucial role in controlling the drug release for anomalous transport, non-Fickian diffusion.

drug release mechanism. The initial burst release was less than the design A as the drug was shielded by albumin layer.

3.2.4.3. Design C. The formulation design C consists of AMB attached to the IONP cores and then the albumin was bound onto it, followed by binding of AMB onto the albumin. The drug release profile was showed in **Figure 3-18**, which indicates a controlled release profile with up to ~75% of cumulative drug was released in first 72 hours. Upon study of the release kinetics the data indicated that the first order and Higuchi models as best fit for release kinetics with r^2 of 0.6936 and 0.6715, respectively (**Figure 3-19**), which concludes that the drug released via diffusion controlled mechanism and the drug release rate was dependent on the concentration of drug in design C formulation of AMB-IONP. For the release mechanism study with Korsmeyer-Peppas release model exhibited high correlation for drug release from design C formulation at pH 7.4 (**Figure 3-20**). The diffusion exponent, n was about 0.47, which indicates the Fickian diffusion was the controlling factor of drug release mechanism.

3.2.4.4. Design D. The formulation design D prepared by attaching mixture of AMB bound albumin onto the IONP cores. In contrast to other designs the study was conducted in 1x PBS containing 4% albumin at pH 7.4. The drug release profile was exhibited in **Figure 3-21**, which indicates a sustain drug release profile over the period of 73 hours with cumulative drug release of 76%. Upon study of the release kinetics the data indicated that it followed first order and Higuchi models as best fit for release kinetics with r^2 of 0.9614 and 0.9665, respectively (**Figure 3-22**), which concludes that the drug released via diffusion controlled mechanism and the drug release rate was dependent on the concentration of drug in design D formulation of AMB-IONP. For the release mechanism study with Korsmeyer-Peppas release model exhibited high correlation for drug release from design D formulation at pH 7.4 (**Figure 3-23**). The diffusion exponent, n was about 0.26, which indicates the Fickian diffusion was the controlling factor of drug release mechanism.

In an attempt to further control the drug release mechanism the formulation design D was subjected to albumin cross linking with glutaraldehyde. The drug release profile was exhibited in **Figure 3-21**, as expected the drug release was further controlled and found to be following sustain release kinetics with only 64% of cumulative drug released within first 73 hours. Upon study of the release kinetics the data indicated that it followed first order first order and Higuchi models as best fit for release kinetics with r^2 of 0.9643 and 0.9690, respectively (**Figure 3-24**), which concludes that the drug released via diffusion controlled mechanism and the drug release rate was dependent on the concentration of drug in the formulation. For the release mechanism study with Korsmeyer-Peppas release model exhibited good correlation for drug release from design D cross linked with glutaraldehyde at pH 7.4 (**Figure 3-25**). The diffusion exponent, n was about 0.50, which indicates the Fickian diffusion was the controlling factor of drug release mechanism. The cross linking of albumin with glutaraldehyde has given rigid structure that delay the diffusion of the drug from the nanoparticles. Hence, a more

Figure 3-18. Cumulative % drug release from design C

The data were presented as mean $\pm$ SD (n=4). The study was conducted in release media of 1x PBS at 37°C. Sustained release of drug from AMB-IONP formulation design C was observed for about 72 hours.

Figure 3-19. Drug release model fit for design C

The best fit for drug release kinetic model was found to be first order and Higuchi with r^2 of 0.6936 and 0.6715, respectively. This concludes that the drug released via diffusion controlled mechanism and the drug release rate was dependent on the concentration of drug in design C formulation of AMB-IONP. Through diffusion drug slowly released from the surface.

Figure 3-20. Korsmeyer-Peppas model fit determination of drug release mechanism from design C

The diffusion exponent, n was found to be 0.47 indicating Fickian diffusion as controlling factor of drug release mechanism. This could be resulted from the internal stress applied by one part of the medium on another as diffusion takes place. The double layer of AMB may play a crucial role in controlling the drug release for Fickian diffusion. One layer of AMB is adsorbed to albumin surface, which could diffuse easily, but whereas another layer is inside and bind to the IONP cores may take longer time for release with concentration independent.

Figure 3-21. Cumulative % drug release from design D with and without glutaraldehyde

The data were presented as mean $\pm$ SD (n=3). The study was conducted in release media of 4% BSA in 1x PBS at 37°C. Sustained release of drug from AMB-IONP formulation design D with and without albumin cross linker glutaraldehyde was observed for about 72 hours. As predicted glutaraldehyde formulation exhibited more sustain release profile.

Figure 3-22. Drug release model fit for design D

The best fit for drug release kinetic model was found to be first order and Higuchi with r^2 of 0.9614 and 0.9665, respectively. This concludes that the drug released via diffusion controlled mechanism and the drug release rate was dependent on the concentration of drug in design D formulation of AMB-IONP. Through diffusion drug slowly released from the surface.

Figure 3-23. Korsmeyer-Peppas model fit determination of drug release mechanism from design D

The diffusion exponent, n was found to be 0.26 indicating Fickian diffusion as controlling factor of drug release mechanism. Here, the possibility of drug is on surface can easily diffuse into the medium. Fickian diffusion is dependent on concentration gradient and surface area of the particles.

Figure 3-24. Drug release model fit for design D cross linked with glutaraldehyde

The best fit for drug release kinetic model was found to be first order and Higuchi with r^2 of 0.9643 and 0.9690, respectively. This concludes that the drug released via diffusion controlled mechanism and the drug release rate was dependent on the concentration of drug in the formulation. Through diffusion drug slowly released from the surface.

Figure 3-25. Korsmeyer-Peppas model fit determination of drug release mechanism from design D cross linked with glutaraldehyde

The diffusion exponent, n was found to be 0.50 indicating Fickian diffusion as controlling factor of drug release mechanism. This could be resulted from the internal stress applied by one part of the medium on another as diffusion takes place. The cross linker glutaraldehyde may play a crucial role in controlling the drug release for Fickian diffusion.

sustain release kinetics was observed.

3.2.5. Colloidal stability in FBS

Stability determination of AMB-IONP formulation (design D) in serum was considered essential for their *in vivo* usage where dilution after intravenous administration could result in nanoparticulate aggregation, disassembly and/or drug leakage. AMB-IONP were incubated in FBS (50 %) at 37°C with gentle shaking. The samples were then analyzed 560 nm absorbance. Absorption method was used since AMB-IONP cannot be accurately detected in dense serum solution by DLS technique due to the matrix protein interference from serum [241]. Aggregation phenomena were characterized by increased absorbance to higher values. The interaction of AMB-IONP with various serum proteins in the incubation media could lead neutralization of ζ -potential of nanoparticles. The process potentially could cause the reduction of repulsion energy among nanoparticles and gradually facilitate agglomeration process, which could lead to the formation of larger particles. AMB-IONP found to be stable in serum retaining the integrity up to 24 hours as observed in **Figure 3-26**. The data also indicates no significant change in absorbance values at 560 nm.

3.3. Conclusions

The formulation designs by changing the order of the layers of AMB and BSA in the development of AMB-IONP were identified and optimized. The albumin was successfully attached to the nanoparticles after following the standard protocol for binding the amine containing molecules to the carboxylic acid groups expressed on the oleic acid coated IONP cores. The different formulation designs were developed to increase the drug loading capability, to have the AMB on the surface for the targeting effect and to minimize the processing steps. The approach was successful with design D being the most fruitful in that regard with maximum drug loading of $13.6 \pm 6.9 \mu\text{g}$ of AMB/mg of nanoparticle. The size and ζ -potential for all the formulation designs found to be optimum for a nanomedicine with $\leq 36 \text{ nm}$ and $\sim -20 \text{ mV}$, respectively. The PDI was also found to be optimum with ≤ 0.2 PDI. By changing the order of AMB and BSA did not significantly affect the physical characteristics of the formulation designs, which was also evidenced by TEM imaging. The TEM images confirmed that the nanoparticles before and after formulating the designs were monodispersed and spherical in shape.

The drug release profile indicated a burst release up to 3 hours for design A and B, followed by a sustained drug release profile up to at least 72 hours. The best potential designs C and D indicated a sustain drug release profile up to 72 hours. Design D even in the presence of 4% albumin as a dissolution medium exhibited a sustained drug release profile with and without glutaraldehyde cross linker. The major mechanism of drug release from these formulations found to be Fickian and non-Fickian diffusion with first order and Higuchi kinetic models as best fit. The design D formulation was stable in 50% serum for up to 24 hours. The unique nature of this parenteral formulation has the

Figure 3-26. Physical stability of design D formulation of AMB-IONP in 50% FBS

The data were presented as mean $\pm$ SD (n=3). Particles retained their physicochemical properties without aggregation for a prolonged period of time (24 hours).

potential to overcome the barriers in chemotherapy of drugs for deadly fungal infections.

CHAPTER 4. *IN VITRO* EVALUATION OF CELLULAR UPTAKE AND EFFICACY

In vitro studies have been extensively used for evaluating new nanomedines, particularly cell culture modes [242]. These studies offer economical and potential means of evaluating many properties when compared to expensive animal studies. The largest advantage is that *in vitro* cell based studies are a proven exclusive platform to evaluate the safety and efficacy of nanoparticulate drug delivery systems under well controlled and reproducible settings [243]. These studies allow a number of experimental variables, which can be manipulated to mimic the *in vivo* circumstances [244].

For any antifungal drug to show efficacy, it has to get to and/or into the cells of the causative organism. So, the first evaluation step is to determine the cellular uptake in fungal clinical isolates. This is evaluated by performing both qualitative and quantitative tests of cellular uptake using fluorescent labeled nanoparticles. Qualitative uptake and visualization of fluorescent labeled AMB-IONP was investigated by imaging using a confocal laser scanning microscopy method [242]. The quantitative evaluation of cellular uptake fluorescent labeled AMB-IONP was studied by flow cytometry analysis [245]. These two techniques are commonly used to determine the cellular uptake of molecules or particles [246-249].

With the increased incidence of IFIs the demand for novel antifungal drugs is growing and the selection of antifungal treatment choices have gained more attention [197]. The Clinical and Laboratory Standards Institute (CLSI) provides validated reference standard method (M27-A3, a broth macrodilution method) for testing the susceptibility to antifungal agents of yeasts, such as the pathogens from the *Candida* species. These species have potentiality to cause IFIs [197]. Susceptibility testing are considered important in an attempt of developing new antimicrobial drugs [250]. Susceptibility testing techniques allows the ability to evaluate comparative therapeutic efficacy of drug loaded AMB-IONP against the conventional AMB-D.

Minimal inhibitory concentration (MIC) is the lowest antifungal agent concentration required to exhibit a specified reduction in visible growth in an agar or broth dilution susceptibility experiment. The MIC determined through these experiments can guide physicians about the required minimum antifungal agent concentration at the infection site to inhibit the infecting pathogen [250, 251]. However, the MIC is not an accurate concentration and the true *in vitro* MIC often lies between the obtained MIC that inhibits the organism's growth and the next lower test concentration. Usually, the end points for AMB are well defined and the MIC is easily read as the lowest AMB concentration that inhibits any discernible growth of fungal species tested [197]. It's unusual to see trailing end points with AMB.

The objective of the work described was to determine the AMB-IONP uptake in *C. albicans* and *C. glabrata* clinical isolates and to evaluate its *in vitro* efficacy along with AMB-D against clinical isolates of *Candida* species and compare the MIC values.

4.1. Materials and Methods

4.1.1. Materials

The fungal clinical isolates *Candida albicans* (SC5314), *Candida glabrata* (66032) were obtained from American Type Culture Collection (Manassas, VA, USA). *Candida krusei* (CK1), *Candida parapsilosis* (CP1), and *Candida tropicalis* (CT1) were shared by Dr. Daniel J. Diekema at the University of Iowa (Iowa City, IA, USA). The AMB-D was purchased from X-GEN Pharmaceuticals, Inc. (Horseheads, NY, USA). DMSO and Coumarin 6 dye was obtained from Sigma-Aldrich (Saint Louis, MO, USA). PBS and HPLC grade water purchased from Fisher (Pittsburg, PA, USA). LysoTracker® Deep Red was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Vectashield® cell mounting medium with 4', 6-diamidino-2-phenylindole (DAPI) was acquired from Vector Labs (Burlingame, CA, USA). RPMI (Roswell Park Memorial Institute) 1640 was obtained from Sigma (St. Louis, MO, USA).

4.1.2. Preparation of fluorescently labeled AMB-IONP for imaging

The AMB-IONP formulation of design D and control without drug were prepared with the same technique described in Chapter 3. For imaging cellular uptake and intracellular localization in fungal clinical isolates, fluorescent labeled AMB-IONP were prepared by mixing particles with Coumarin 6 dye dissolved in DMSO for 2 hours. Coumarin 6 is a green fluorescent dye. The amount of dye used was 1% by weight of IONP. After adsorption of dye on to the particles, the free dye was washed with wash/storage buffer and removed with the help of magnetic separation technique previously described in Chapter 3. The step was performed at-least twice to ensure the removal of the free dye. Finally, the fluorescent labeled AMB-IONP were dispersed in wash/storage buffer and stored at 2-8°C until further used.

4.1.3. Cellular uptake of AMB-IONP by confocal laser scanning microscopy

Qualitative uptake and visualization of design D formulation of AMB-IONP were investigated by imaging through a confocal laser scanning microscopy method [242]. 600 µL of *C. albicans* and *C. glabrata* clinical isolates at a cell density of 10^4 /mL were placed in each well for each time point. The cells were grown for an additional one day to establish enough cell density for the study performed at 0.5 and 4 hour time points. Both isolates were treated with the formulations at each time point. Fifteen minutes before end of the time point, each well was treated with LysoTracker® deep red (50 nM), a unique marker for secondary endosomes and lysosomes. Following the incubation period, isolates were washed three times with 1x PBS and centrifuged at 13000 rpm for 15 minutes. The pellet formed at the end of third wash was treated with one drop of Vectashield® cell mounting medium with DAPI to visualize nuclei as blue fluorescent shapes. The mixture was mixed well with a pipette and then placed on microscope slide

and covered with coverslip. The slide was dried in a fume hood for 15-20 minutes. After drying the sides of the coverslip were immobilized to the slide with the help of clear nail polish. The slides were again allowed to dry for another 15-20 minutes and stored in freezer until further usage. The slides were then observed with a Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss SMT Inc., USA) by using a Plan-Apochromat 60×/1.40 na Oil DIC objective lens. The obtained images were processed with the help of Zeiss LSM Zen software 2010.

4.1.4. Cellular uptake of AMB-IONP by flow cytometry

The quantitative evaluation of cellular uptake, flow cytometry method was used [245]. Both the isolates of *C. albicans* and *C. glabrata* were prepared and treated in a similar way as for the confocal imaging studies. The uptake was studied at 0, 0.5, 1, 2, and 4 hours. At the end of three washes with 1x PBS, the pellet was re-dispersed in 600 µL of 1x PBS. Each sample was then subjected to flow cytometry analysis to determine mean fluorescence intensity (MFI) with the help of BD Accuri™ C6 flow cytometer (Becton Dickinson Inc., NJ, USA) with FL1-A channel. Each sample was run in triplicate and the obtained data was processed with BD Accuri™ CFlow Plus analysis software.

4.1.5. *In vitro* efficacy of AMB-IONP in clinical isolates of *Candida*

Susceptibility testing control, AMB-D and all formulation designs (A through E) of AMB-IONP were performed using a microbroth dilution assay according to the CLSI guidelines outlined in M27-A3 with a few modifications [197]. Isolates tested include *C. albicans* SC5314, *C. glabrata* 66032, *C. krusei* CK1, *C. parapsilosis* CP1, and *C. tropicalis* CT1. Additionally, *C. albicans* CA1008 and Calowa60, which contains less than 1% ergosterol were also used for testing. Cultures were streaked on Sabouraud Dextrose plates and allowed to grow for 24 hours. Colonies were then picked into sterile water and diluted to an optical density of 0.1 when measured at 600 nm. Cultures were diluted to a final inoculum of 0.5- 2.5×10³ cells/mL in RPMI 1640 with 2% glucose, MOPS (3-(N-morpholino)propanesulfonic acid, 4-morpholinepropanesulfonic acid), pH 7.0. Ninety six well plates were incubated with increasing concentrations of the test compounds or controls at 35°C for 48 hours. Absorbance at 600 nm was read with a microplate reader (Biotek, Winooski, VT, USA). Background absorbance due to medium was subtracted from all readings. The MIC was defined as the lowest concentration that prevented any discernible growth. IC₉₀ were calculated using SigmaPlot 12.0 at 24 hours of treatment. Heat maps were prepared using Java TreeView. In addition, preliminary studies including transferrin as a targeting ligand and curcumin as a second drug load were also conducted with design D against clinical isolates of *C. albicans* SC5314, *C. glabrata* 66032, *C. krusei* CK1, *C. parapsilosis* CP1, and *C. tropicalis* CT1. After preparation of design D of AMB-IONP, the particles were dispersed in 100 µL of transferrin solution (100 µg/mL) in water and incubated at ambient temperature for 2 hours, followed with washing the unbound transferrin with similar washing steps involved for other formulations.

In order to test whether multiple drugs can be loaded on to the formulation design D, curcumin was loaded along with AMB and subjected to susceptibility testing. AMB and curcumin have different binding domains on HSA and BSA and AMB retains its antifungal activity [252]. In addition, curcumin can potentially reduce the toxic side effects as it will delay the lysis of the red cells by AMB.

4.2. Results and Discussion

4.2.1. Cellular uptake of AMB-IONP

Both the qualitative and quantitative analysis of the uptake of AMB-IONP formulation design D was determined with confocal laser scanning microscopy and flow cytometry, respectively. Confocal microscopy was employed to visualize the localization of nanoparticles in the cellular compartments, while flow cytometry was employed to determine the MFI for the clinical isolates of *C. albicans* and *C. glabrata* treated with formulations. The AMB-IONP were labeled with coumarin 6 dye by 1% weight of IONP for visualization and MFI determination. The fluorescent labeled AMB-IONP were also subjected for physicochemical characterization (size, PDI, and ζ -potential) and the results found to be similar to that of non-fluorescent labeled AMB-IONP (data not presented).

The images of confocal microscopy for the cellular uptake of design D formulation of AMB-IONP in the clinical isolates of *C. albicans* and *C. glabrata* were represented in **Figure 4-1** and **Figure 4-2**, respectively. Coumarin 6 (indicative of AMB-IONP), LysoTracker[®] (indicative of secondary endosome and lysosome), and DAPI (indicative of nuclei) fluoresce green, red, and blue, respectively. The confocal microscopy revealed that the AMB-IONP were co-localized with endosomes and lysosomes and widely existing in the cytoplasm of the both isolates. For *C. albicans* 0.5 hour exhibited significantly higher uptake when compared to 4 hour time point. Whereas, for *C. glabrata* 4 hour time point exhibited significantly higher uptake in comparison to 0.5 hour time point.

Flow cytometry revealed the time dependent cellular uptake of nanoparticles in both the isolates tested. The MFI indicated maximum uptake within first 30 minutes after treatment for *C. albicans* as shown in the **Figure 4-3**. The uptake plateaued between 0.5 and 1 hour after treatment. The *C. glabrata* isolate showed maximum uptake was at the 4 hour time point as shown in the **Figure 4-4**. The uptake plateaued between 2 and 4 hours after treatment. These results were in good agreement with confocal microscopy data. Thus, the confocal microscopy and flow cytometry results confirm the uptake of AMB-IONP in the both the isolates tested.

Figure 4-1. Confocal scanning laser microscopy images of cellular uptake of design D in *C. albicans*

The nucleus was stained blue with DAPI. The images indicate the endolysosomal localization with colocalization of AMB-IONP (green) and lysotracker in red in the merged image and time dependent uptake of nanoparticles in green, with a maximum uptake at 0.5 hour. The scale bar represents 2 microns.

Figure 4-2. Confocal scanning laser microscopy images of cellular uptake of design D in *C. glabrata*

The nucleus was stained blue with DAPI. The images indicate the endolysosomal localization with colocalization of AMB-IONP (green) and lysotracker in red in the merged image and time dependent uptake of nanoparticles in green, with maximum uptake at 4 hours. The scale bar represents 1 micron for 0.5 hours and 2 microns for 4 hours.

Figure 4-3. Flow cytometry study of cellular uptake of design D in *C. albicans* clinical isolate

The MFI indicated maximum uptake between 0.5 and 1 hour. The data is presented as mean $\pm$ SD (n=3).

Figure 4-4. Flow cytometry study of cellular uptake of design D in *C. glabrata* clinical isolate

The MFI indicated maximum uptake between 2 and 4 hours. The data is presented as mean $\pm$ SD (n=3).

4.2.2. *In vitro* efficacy of AMB-IONP fungal clinical isolates of *Candida* species

Susceptibility testing was performed once the cellular uptake of these developed AMB-IONP formulations was confirmed. Representative heat maps and dose response curves for the susceptibility testing are shown in **Figure 4-5** and **Figure 4-6**, respectively. The control formulation that contains no AMB showed no efficacy or toxicity towards the fungal clinical isolates tested. This indicates the amount of IONP used can be considered safe for the study. The IC₉₀ values calculated at the end of 24 hours of the study indicated all the formulation designs of AMB-IONP had better killing power over AMB-D against *C. albicans* (**Figure 4-7**), *C. glabrata* (**Figure 4-8**), *C. krusei* (**Figure 4-9**), *C. parapsilosis* (**Figure 4-10**), and *C. tropicalis* (**Figure 4-11**). Among the developed formulations, design D was found to be the lead formulation with 16-25 fold more efficacious in eradicating 90% of any discernible growth when compared to marketed conventional product AMB-D. There could be many reasons for better efficacy of the developed formulation designs, particularly for lead design D, which was also found to be lead in the amount of drug loading. First, these nanoparticles are extremely small (30-40 nm) for nanomedicine and have shown higher cellular uptake due to targeting effect or due to the size when compared to AMB-D micellar product. Secondly, the albumin bound AMB to the BSA can act as targeting agent as well as an anti-infective due its higher binding affinity towards the ergosterol present in the fungal cell membranes. Additionally, small particles with ≤ 40 nm with large surface area and albumin coating as a targeting agent can potentially have an additive effect producing a higher cellular uptake. Since each particle is loaded with significantly high payload of AMB, which is delivered at/inside the fungal cell may explain the relatively higher killing effectiveness. More AMB means better chance for wider pores for higher efficiency in killing fungal cells [16]. The additive effect of size in terms of cellular uptake can also be seen in the fungal isolates of *C. albicans* CA1008 and Calowa60, which contains less than 1% of ergosterol. Formulation design D was found to be more efficacious than AMB-D, but overall required twice the AMB amount to kill the fungal cells with low ergosterol (data not presented). This also confirms the targeting ability of the AMB on formulation designs for other fungal clinical isolates tested. Overall, these results confirm the lead formulation was design D with up to 25-fold more efficacious than AMB-D.

The transferrin bound design D formulation has significantly lower IC₉₀ values over AMB-D for clinical isolates tested (**Figure 4-12**). These results indicate that transferrin can also potentially be used as targeting ligand, particularly for targeting CNS, where transferrin receptors are overexpressed during inflammation or tumor [26]. Similar results were observed with dual drug loading formulation with AMB and curcumin (**Figure 4-13**). The outcome from this study suggests that this formulation design could be used to load multiple drugs that have different binding domains on the albumin.

Figure 4-5. Representative heat maps for susceptibility testing of indicated *Candida* isolates

For control without AMB (IONP) the concentrations indicate the same amount of particles treated for other formulation designs. Each well was normalized to the no drug growth control. The data is representative of at least two reproducible experiments.

Figure 4-6. Dose response curves for treatment of clinical isolates of the indicated species of *Candida* (n=3)

Cell densities were measured at 48 h and normalized to the control wells containing no drug.

Figure 4-7. IC₉₀ values estimation for susceptibility testing of *C. albicans* clinical isolate

The data presented as geometric mean for AMB-D, design A, B, C, D, and E with n=3, 3, 2, 5, 3, and 2, respectively. The IC₉₀ value for control formulation (IONP without AMB) found to be >3 µg/mL. Refer to section 4.2.2 for discussion of the results.

Figure 4-8. IC₉₀ values estimation for susceptibility testing of *C. glabrata* clinical isolate

The data presented as geometric mean for AMB-D, design A, B, C, D, and E with n=3, 3, 2, 5, 3, and 2, respectively. The IC₉₀ value for control formulation (IONP without AMB) found to be >3 µg/mL. Refer to section 4.2.2 for discussion of the results.

Figure 4-9. IC₉₀ values estimation for susceptibility testing of *C. krusei* clinical isolate

The data presented as geometric mean for AMB-D, design A, B, C, D, and E with n=3, 3, 2, 5, 3, and 2, respectively. The IC₉₀ value for control formulation (IONP without AMB) found to be >3 µg/mL. Refer to section 4.2.2 for discussion of the results.

Figure 4-10. IC₉₀ values estimation for susceptibility testing of *C. parapsilosis* clinical isolate

The data presented as geometric mean for AMB-D, design A, B, C, D, and E with n=3, 2, 2, 5, 3, and 2, respectively. The IC₉₀ value for control formulation (IONP without AMB) found to be >3 µg/mL. Refer to section 4.2.2 for discussion of the results.

Figure 4-11. IC₉₀ values estimation for susceptibility testing of *C. tropicalis* clinical isolate

The data presented as geometric mean for AMB-D, design A, B, C, D, and E with n=3, 2, 2, 5, 3, and 2, respectively. The IC₉₀ value for control formulation (IONP without AMB) found to be >1 µg/mL. Refer to section 4.2.2 for discussion of the results.

Figure 4-12. IC₉₀ values estimation for susceptibility testing of indicated *Candida* clinical isolates treated with AMB-D and transferring (Tf) bound design D (n=1)

Refer to section 4.2.2 for discussion of the results.

Figure 4-13. IC₉₀ values estimation for susceptibility testing of indicated *Candida* clinical isolates treated with AMB-D and dual drug loading of AMB and curcumin (CUR) in design D (n=1)

Refer to section 4.2.2 for discussion of the results.

4.3. Conclusions

In vitro experiments for the determination of cellular uptake and the susceptibility testing were conducted. The flow cytometry results for cellular uptake of formulation design D in *C. albicans* and *C. glabrata* clinical isolates exhibited time dependent uptake. The cellular uptake reached a plateau between 0.5 and 1 hour and between 2 and 4 hours for *C. albicans* and *C. glabrata*, respectively. These results were in good agreement with confocal scanning laser microscopy images of formulation design D uptake. All the formulation designs of AMB-IONP exhibited improved efficacy over marketed AMB-D product in the susceptibility testing conducted in clinical isolates of *C. albicans* SC5314, *C. glabrata* 66032, *C. krusei* CK1, *C. parapsilosis* CP1, and *C. tropicalis* CT1. Design D was found to be superior and was 16-25 fold more efficacious than the conventional product (AMB-D). Even in clinical isolates of *C. albicans* CA1008 and Calowa60 that has < 1% of ergosterol, design D had better killing power over AMB-D. The initial studies with transferrin as a targeting ligand bound to the surface of design D formulation also exhibited improved efficacy over AMB-D for isolates tested. Therefore transferrin could be an alternative targeting ligand, particularly for fungal meningitis, which is overexpressed with transferrin receptors during infection. Additionally, this formulation design could also be used to load multiple drugs that have different binding regions on BSA or HSA such as AMB and curcumin. With these results, I propose that AMB-IONP may potentially serve as a better and more efficacious drug delivery system over conventional marketed product AMB-D for IFIs.

CHAPTER 5. *IN VITRO* ANALYSIS OF UPTAKE MECHANISM AND INTRACELLULAR TRAFFICKING

For infectious diseases, the nature of the pathogen and body responses dictate the choice of drug substance, route of administration, absorption, distribution, metabolism, excretion and dosing regimen [5, 12, 14, 190]. In Chapter 5, I discussed the experiments that affect drug action on the pathogenic fungi. Nanomedicine particles are handled differently by the body than solution drugs and there is a need to focus on the uptake mechanisms and intercellular trafficking of the nanomedicine particles. In contrast drugs in true solution often enter cells by different mechanisms such as passive diffusion, active transport or receptor activation. Additionally, solution drugs may be rapidly processed once inside the pathogen. To highlight these differences this section contains information characterizing how the newly designed nanomedicine containing AMB interacts with selected fungal pathogens.

Better knowledge of nanoparticles properties and the experimental conditions that determine the uptake and transport across cell membranes will improve understanding of their uptake mechanism and may be beneficial in developing safer and more efficacious nanomedicines [253]. Knowing the uptake pathway of the nanoparticles in different fungal isolates and the design properties that affect the localization in the cell organelles, will be useful in designing and predicting the drug delivery system's actions.

The cellular uptake mechanisms and trafficking of nanoparticles are strongly influenced by particle properties such as size [254, 255], shape [256, 257], surface charge [258, 259], surface functional groups [260] and hydrophilicity [261]. Cell membranes are usually impermeable to many large particles [253, 262]. Commonly the small nanoparticles in the size range of 10-30 nm have potential to cross the cell membrane and have access to intracellular organelles [263]. Endocytosis is a process to overcome the cell membrane barrier. This process is often exploited by viruses to penetrate cells [253]. Different particle cellular uptake pathways are depicted in the **Figure 5-1** [264]. The proposed cellular uptake mechanisms for small nanoparticles are types of endocytosis and diffusion. Nanomedicine sized nanoparticles are a similar size range to viruses and some are known to use similar pathways to enter pathogens. Pinocytosis is a fundamental and vital uptake pathway, which is also one type of endocytosis [265]. The endocytosis uptake mechanism is carried out by all eukaryotic cells and the major uptake pathway is a clathrin-mediated endocytosis [266, 267]. Receptor-mediated endocytosis is well investigated mechanism and plays an crucial part in the understanding of nonphagocytic uptake pathways [253]. This process permits a variety of ligands and include cholesterol-carrying proteins, vitamins, toxins, iron transport proteins to penetrate cells through the binding of specific receptors residing on the cell membrane [266, 268]. Receptor-mediated endocytosis is also found to be a common uptake mechanism for eukaryotic cells [269]. Hence, endocytosis uptake mechanisms are ubiquitous in eukaryotic cells. Basically, endocytosis is the uptake of particulate matter including proteins into eukaryotic cells by cell membrane enclosure.

Figure 5-1. Schematic representation of different cellular uptake pathways

Uptake of large size particles usually occurs by phagocytosis, while fluid is taken up by the macropinocytosis pathway. Both pathways are commonly initiated by and are reliant on actin-mediated transformation of the plasma membrane at a large scale. The vesicle size formed by these processes are much larger when compared to the vesicles formed by other endocytic pathways. Clathrin-mediated endocytosis is a receptor-mediated endocytosis process through the formation of clathrin coated pits. Caveolae-mediated endocytosis is also a receptor mediated endocytosis process with formation of invaginated, vesicular/tubulovesicular or flask shaped vesicles, known as caveolae. Clathrin- and caveolin-independent endocytosis have vesicle formation of about 50 nm. These pathways require specific lipid compositions, such as cholesterol or ergosterol (in case of yeast cells). Various cargoes can be endocytosed by pathways, which are independent of the clathrin (coat protein) and dynamin (fission GTPase). The majority of the internalized cargoes are transported to the early endosome through clathrin- or caveolin-coated vesicles (vesicular) or clathrin- and dynamin independent carriers (CLICs) (tubular intermediates), which are derived from the plasma membrane. Before trafficking to the early endosome, some mechanisms may first traffic to intermediate compartments and include caveosome or glycosyl phosphatidylinositol-anchored protein enriched early endosomal compartments (GEEC).

Pinocytosis, can be classified into several uptake processes based on the size of the vesicle and the protein involved in formation of the vesicles. The mechanisms can be detailed as follows: 1) Macropinocytosis is a nonspecific uptake pathway where the fluid components are engulfed in the same concentrations as of the surrounding medium [253]. The vesicles formed are a size of 0.1 to 5 microns [270]. The morphology of this pathway is highly ruffled formed by actin cytoskeleton [271]. 2) Clathrin-mediated endocytosis is a receptor-mediated endocytosis process through the formation of clathrin coated pits with vesicular morphology size in the range of 35-60 nm for yeasts [267, 271]. 3) Caveolae-mediated endocytosis is also a receptor mediated endocytosis process with formation of invaginated, vesicular/tubulovesicular or flask shaped vesicles, known as caveolae, with size of approximately 60-80 nm [270, 271]. These vesicles are enriched with cholesterol and sphingolipids, internalizing through caveolae- or lipid raft mediated vesicles, which migrate to caveosomes. Internalized cargo in caveosomes could reside or migrated to the Golgi complex or to the endoplasmic reticulum. 4) Clathrin- and caveolin-independent endocytosis have vesicle formation of about 50 nm. These pathways require specific lipid compositions, such as cholesterol or ergosterol (in case of yeast cells) dependent pathway [271, 272]. Lipid-raft mediated endocytosis is an endocytic pathway, which is sensitive to membrane lipid sterols perturbation [273].

In addition to cellular uptake pathways, the intracellular trafficking of drug loaded nanoparticles and bioavailability of the transported drug within the fungal cell are important elements to investigate when a novel drug delivery system is proposed [274]. In addition to the quality, safety, and efficacy of nanoparticulate drug delivery systems, the nanomedicines need to be extensively studied to fulfill the guidelines written and approved by regulatory organizations including the US Food and Drug Administration. *In vitro* studies have been extensively used for evaluating nanomedicines, particularly cell culture modes, which are considered remarkably economical and potential means in comparison to more expensive animal studies [242]. These *in vitro* cell based studies are a platform to evaluate the safety and efficacy of nanoparticulate drug delivery systems under well controlled and reproducible settings [243]. These studies also offer a number of experimental variables, which can be easily manipulated to mimic the *in vivo* circumstances such as cytokines, growth factors, carbohydrates, and pH [244].

It is proposed that AMB-IONP may serve as an effective drug delivery system for IFIs. Therefore, the goal of this work was to study the uptake mechanism and intracellular trafficking of AMB-IONP (design D) in fungal clinical isolates of *C. albicans* and *C. glabrata*.

5.1. Materials and Methods

5.1.1. Materials

Amiloride, chlorpromazine, genistein, methyl- β -cyclodextrin (M β CD), nocodazole, and sucrose were acquired from Sigma-Aldrich (St. Louis, MI, USA).

LysoTracker® Deep Red was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Vectashield® cell mounting medium DAPI was acquired from Vector Labs (Burlingame, CA, USA). TEM sample preparation supplies were kindly offered by the UTHSC Neuroscience Institute (Memphis, TN, USA). The fungal clinical isolates *Candida albicans* (SC5314), *Candida glabrata* (66032) were obtained from American Type Culture Collection (Manassas, VA, USA). *Candida krusei* (CK1), *Candida parapsilosis* (CP1), and *Candida tropicalis* (CT1) were shared by Dr. Daniel J. Diekema at the University of Iowa (Iowa City, IA, USA).

5.1.2. Cell association study of fluorescently labeled AMB-IONP

The uptake mechanism of design D particles of AMB-IONP into *C. albicans* and *C. glabrata* clinical isolates was determined by conducting receptor mediated endocytosis cellular association studies at 4°C and 35°C [275, 276]. The fluorescently labeled particles and cells for *in vitro* study were prepared as reported in Chapter 4. The study was performed in two separate cell culture plates maintained at 35°C and pre-cooled to 4°C, respectively before the treatment. The cells were treated and incubated for 1 hour at a final concentration of 0.02 µg/mL of AMB. The cells were then centrifuged and the pellet was washed thrice with 1x PBS and finally redispersed in 0.6 mL of 1x PBS and the MFI was calculated per 10,000 cells using a BD Accuri™ C6 flow cytometer with FL1-A channel. Each sample was run in triplicate and the data was evaluated using BD Accuri™ CFlow Plus analysis software.

5.1.3. The mechanisms of cellular uptake of AMB-IONP into fungal isolates

The potential cellular uptake pathways of fluorescent design D particles was determined for clinical isolates of *C. albicans* and *C. glabrata*. The fluorescently labeled particles and cells for *in vitro* study were prepared as reported in Chapter 4. The cells were pre-treated with endocytosis inhibitors such as amiloride, chlorpromazine, genistein, MβCD, and nocodazole as per reported methods [277-280]. These inhibitors were added to inhibit macropinocytosis (amiloride), clathrin-mediated endocytosis (chlorpromazine), caveolae-mediated endocytosis (genistein), lipid-raft-mediated endocytosis (MβCD) and microtubule-dependent endocytosis (nocodazole). The study was conducted at 35°C. The cells were incubated with inhibitors for one hour, followed with treatment of fluorescently labeled AMB-IONP for one hour. The control samples were prepared without using inhibitors. Cells were then centrifuged and the pellet was washed thrice with 1x PBS. At the end of three washes the pellet was re-dispersed in 600 µL of 1x PBS. Each sample was subjected to flow cytometry analysis to determine MFI per 10,000 cells with the help of BD Accuri™ C6 flow cytometer with FL1-A channel. Each sample was run in triplicate and the data was processed with BD Accuri™ CFlow Plus analysis software. In addition, the uptake pathway was also observed with TEM and the sample preparation was the same as for intracellular trafficking studies.

5.1.4. Intracellular trafficking of AMB-IONP by TEM

One of the most promising ways of interpreting the intracellular localization of nanoparticles in yeast cells is by TEM [281]. The design D of AMB-IONP formulation was used against *C. albicans* and *C. glabrata* clinical isolates. Both isolates were treated with nanoparticles and incubated for 4 hours at 35°C. Following the incubation period, isolates were washed three times with ice cold 1x PBS and centrifuged at 13000 rpm for 15 minutes. The pellet formed at the end of third wash was redispersed in 500 µL of 2.5% gluteraldehyde (electronic microscope grade) in 1x PBS and incubated overnight at 4°C. Upon the fixation of cells, the dispersion was washed thrice with 1x PBS at 200 g for 10 minutes each. At the end of third wash the cells were stained with 4% osmium tetroxide in 1x PBS for 1 hour and washed for an additional three times with 1x PBS and two times with distilled water. The cells were then dehydrated with 50, 70, 85, and 95% ethanol for 30 minutes each wash. The final dehydration was done three times with 100% ethanol. The cells were then subjected to infiltration in 1:1 ration Spurr's resin and 100% ethanol, and rotated overnight. The infiltration was continued for additional 2 hours in 100% Spurr's resin for three times and the pellet was formed with centrifugation at 800 g for 15 minutes. The cells were then embedded in fresh Spurr's resin in a casting mold and cured at 60-70°C for 48 hours. The cured block was placed in an ultramicrotome and sections of about 70 nm thick were cut with a diamond knife. Chloroform was used to smooth the sections. The sections were scooped from the knife water bath with copper 200 mesh grid and placed on filter paper until dry. The sections were stained with uranyl acetate and lead citrate to increase the contrast and electron density. The grids were observed and imaged on a JEOL 2000EX TEM (Peabody, MA, USA).

5.1.5. Intracellular trafficking of AMB-IONP by confocal microscopy

In order to confirm the TEM results that the IONP were observed in endolysosomes, cells were marked with a endolysosome specific dye (LysoTracker® deep red) [282, 283]. Briefly, 600 µL of *C. albicans* and *C. glabrata* clinical isolates at a cell density of 10^4 /mL were placed in each well for each time point. The cells were grown for an additional one day to establish enough cell density for the study. This study was performed at the 4 hour time point. Both of the isolates were treated with the design D formulation. Fifteen minutes before the end of the time point, each well was treated with LysoTracker® deep red (50 nM), a unique marker for secondary endosomes and lysosomes. Following the incubation period, isolates were washed three times with 1x PBS and centrifuged at 13000 rpm for 15 minutes. The pellet formed at the end of the third wash was treated with one drop of Vectashield® cell mounting medium with DAPI to visualize nuclei as blue fluorescent in the images. The mixture was mixed well with a pipette and then placed on a microscopic slide and covered with a coverslip. The slide was dried in a fume hood for 15-20 minutes. After drying, the sides of the coverslip were immobilized to the slide with the help of clear nail polish. The slides were again allowed to dry for another 15-20 minutes and stored in a freezer (-10°C) until further usage. The slides were observed with a Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss SMT Inc., USA) by using a Plan-Apochromat 60×/1.40 na Oil DIC objective lens.

The obtained images were processed with the help of Zeiss LSM Zen software 2010.

5.1.6. Statistical analysis

In order to compare the mean values between the treatment and control groups student's t-test (two tailed) were executed. The data was reported as mean $\pm$ SD and n is the number of replicates. The results with $p < 0.05$ were considered as statistically significant.

5.2. Results and Discussion

5.2.1. Cell association study

The results of cellular association studies at 35°C and 4°C for *C. albicans* and *C. glabrata* clinical isolates are presented in **Figure 5-2** and **Figure 5-3**, respectively. The MFI reflects the uptake of the nanoparticles into the cells. The results indicated that uptake was significantly reduced in both isolates when the study was conducted at 4°C compared to 35°C. Receptor mediated endocytosis is an energy/temperature dependent pathway, which requires ATP for significant function. The reason for reduced uptake at 4° C could be that the uptake mechanism is receptor mediated endocytosis that requires energy. The uptake mechanism at 4° C was significantly reduced and may be stopped due to the receptor mediated nanoparticles being internalized before washing with PBS. These results suggest that the uptake pathway mechanism of AMB-IONP into both *C. albicans* and *C. glabrata* clinical isolates was a receptor mediated endocytosis.

5.2.2. Uptake mechanism of AMB-IONP into cells

The cellular uptake pathway of nanoparticles mediated through endocytosis can include micropinocytosis, clathrin mediated endocytosis, caveolae mediated endocytosis, clathrin/caveolae independent endocytosis, lipid-raft-mediated endocytosis, and uptake through microtubules [277-280, 284]. The flow cytometry results of the effect of endocytosis inhibitors on the uptake of fluorescently labeled AMB-IONP in both clinical isolates of *C. albicans* and *C. glabrata* are presented in **Figure 5-4** and **Figure 5-5**, respectively.

Even though the data indicated a significant difference in MFI of clathrin-mediated endocytosis inhibitor and lipid-raft-mediated endocytosis when compared to the control group, the major pathway for uptake of AMB-IONP into *C. albicans* was determined to be lipid-raft-mediated endocytosis (**Figure 5-4**). Lipid raft endocytosis is defined as an endocytic pathway, which is sensitive to cholesterol discomposure [273]. In yeast eukaryotes cell receptor and fluid phase endocytosis is reliant on the sterol found in the yeast cells, ergosterol. Ergosterol is the major sterol found in the yeast plasma

Figure 5-2. Cell association study of AMB-IONP in *C. albicans*

The MFI reflects the uptake of the nanoparticles into the cells. The results indicate that uptake was significantly reduced in isolate tested when the study was conducted at 4°C compared to 35°C. Receptor mediated endocytosis is an energy/temperature dependent pathway, which requires ATP for significant function. A reason for reduced uptake at 4°C could be that the active transport uptake mechanism is receptor mediated endocytosis. The data were presented as mean $\pm$ SD (n=3). ** indicates p-value of <0.01 .

Figure 5-3. Cell association study of AMB-IONP in *C. glabrata*

The MFI reflects the uptake of the nanoparticles into the cells. The results indicate that uptake was significantly reduced in isolate tested when the study was conducted at 4°C compared to 35°C. Receptor mediated endocytosis is an energy/temperature dependent pathway, which requires ATP for significant function. A reason for reduced uptake at 4° C could be that the active transport uptake mechanism is receptor mediated endocytosis. The data were presented as mean ± SD (n=3). ** indicates p-value of <0.01.

Figure 5-4. Cellular uptake mechanism study in *C. albicans*

The MFI reflects the uptake of the nanoparticles into the cells. The results indicate that uptake was significantly reduced in isolate tested when the cells were incubated with M β CD before AMB-IONP treatment. This suggests lipid-rafts mediated endocytosis was a major uptake pathway. The data were presented as mean $\pm$ SD (n=3). * indicates p-value of <0.05 and *** indicates p-value of <0.001.

Figure 5-5. Cellular uptake mechanism study in *C. glabrata*

The MFI reflects the uptake of the nanoparticles into the cells. The results indicate uptake was significantly reduced in isolate tested when the cells were incubated with chlorpromazine, genistein, and M β CD before AMB-IONP treatment. This suggests that clathrin mediated endocytosis, caveolae-type endocytosis, and lipid-rafts mediated endocytosis were major uptake pathways. The data were presented as mean $\pm$ SD (n=3). *** indicates p-value of <0.001.

membrane. This strongly suggests a pathway similar to the cholesterol-dependent endocytosis in mammalian cells. This is reinforced by the fact that ergosterol have a greater ability to form lipid rafts than cholesterol [285]. M β CD is an agent used to extract and/or sequester membrane sterols [271, 280]. Since, M β CD inhibited the lipid-raft-mediated endocytosis pathway, very few particles gained entrance into the cell. Therefore, it is concluded lipid-raft-mediated endocytosis is the major pathway for uptake of AMB-IONP into *C. albicans*. It was already known that AMB has greater binding affinity towards the ergosterol found in the fungal cell membrane. Hence, this data also suggests that the AMB is on the surface of the design D formulation of AMB-IONP, which helped target the nanoparticles to the fungal isolate tested and to achieve a more efficacious delivery system over AMB-D. Additionally, the uptake mechanism is also observed with TEM and the image of *C. albicans* cells incubated with AMB-IONP for 4 hours revealed lipid-raft microdomains as seen in **Figure 5-6**. The nanoparticles were found to have adhered to the fungal cell wall to get into the cell membrane, to bind and/extract ergosterol from lipid bilayer of the membrane as per the proposed mechanism of action of AMB. The nanoparticles were present in the cell wall, but TEM could not identify them due to its dense structural composition. The particles also found in abundance at the cell membrane as seen in **Figure 5-6b** indicate the possibility of the lipid-raft mediated endocytosis pathway for the cellular uptake. The morphology of the clathrin- and may be caveolar-independent carriers was found to be similar to the observations reported by M. Kirkham and R.G. Parton [273]. This pathway lacks study and needs to be further characterized in detail. The electron dense particle size of IONP cores was found to be in agreement with the manufacture's specifications (~15 nm). The TEM findings are in agreement and compliment the results of uptake mechanism study with inhibitors.

For the *C. glabrata* clinical isolate, the major uptake pathway of nanoparticles were multiple, and included clathrin-mediated endocytosis, caveolar-type endocytosis, and lipid-raft-mediated endocytosis (**Figure 5-5**). Chlorpromazine is used to block formation of clathrin-coated pits at the plasma membrane and leads to assembly of clathrin lattices on endosomal membranes [286]. The treatment with chlorpromazine exhibited a significant difference in uptake of the particles leading to determination this was a major pathway for endocytosis. Caveolae mediated endocytosis comprises the development of 50-100 nm invaginated plasma membranes, which are identified as caveolae [287]. The main composition of caveolae includes caveolin, glycolipids, and cholesterol. Genistein is a specific inhibitor of caveolae-mediated uptake [288]. Genistein showed a significant inhibition of cellular uptake of AMB-IONP in *C. glabrata* leading to another possible uptake mechanism. The caveolae is formed, when caveolin-1 and caveolin-3 are expressed in non-muscle cells and muscle cells, respectively [273]. It is been recognized as a specific form of lipid raft domain, which has distinctive morphology created by caveolin. It has been found that caveolin-1 directly interacts with membrane sterols and upon membrane sterol depletion leads to disruption of caveolae. Thus, both caveolar-mediated endocytosis and lipid-raft-mediated endocytosis are affected by ergosterol depletion caused by M β CD inhibitor. Together these results suggest that the possibility of the multiple endocytic mechanisms in yeast cells. These findings are in agreement with the literature concerning the possibilities of multiple endocytic

Figure 5-6. Uptake mechanism study with TEM for *C. albicans* treated with AMB-IONP

The particles found in abundance at the cell membrane, in the lipid-raft microdomains indicating the possibility of the lipid-raft mediated endocytosis pathway for the cellular uptake.

mechanisms in yeast cells [273].

Additionally, the uptake mechanism was observed with TEM and the image of *C. glabrata* cells incubated with AMB-IONP for 4 hours revealed clathrin-mediated endocytosis, caveolar-type endocytosis, and lipid-raft-mediated endocytosis as represented in **Figure 5-7**. The first mechanism, clathrin-mediated endocytosis was clearly observed with TEM as the of clathrin-coated structures found with vesicular morphology and the size (~65 nm) of the vesicles are in agreement with the literature [267, 271]. The IONP were found along the peripheral and inside of the vesicles. This interpretation confirmed that the clathrin-mediated endocytosis was one of the cellular uptake pathways for these nanoparticles. The TEM image also provided the evidence of lipid-rafts mediated endocytosis as a second cellular uptake pathway. The particles found in abundance at the cell membrane indicated the possibility of the lipid-raft mediated endocytosis pathway for the cellular uptake. The TEM image also revealed the evidence of caveolae-mediated endocytosis as third uptake mechanism. The caveolae coated pits are found with IONP particles and the morphology was vesicular/tubulovesicular with size of 50-100 nm in diameter, which are in agreement with the literature [289]. The electron dense particle size of IONP cores was also found to be in agreement with manufacture specifications (~15 nm). The TEM findings are in agreement and compliment the results of uptake mechanism study with inhibitors.

Since, there was three possible cellular uptake pathways available for *C. glabrata*, the nanoparticles were infrequently seen on or near the cell wall and cell membrane after 4 hours of incubation when compared to *C. albicans*. Whereas for *C. albicans* there is only one major pathway available for uptake of particles, which could delay the uptake of nanoparticles into the cells. This could be a reason for the better efficacy of design D in *C. glabrata* over *C. albicans*. The MIC data reported in Chapter 4 indicated design D was 9-fold more efficacious than AMB-D in *C. glabrata*, whereas it was only 7-fold more efficacious in *C. albicans* when compared to AMB-D.

5.2.3. Intracellular trafficking of AMB-IONP in fungal clinical isolates

For both clinical isolates of *C. albicans* and *C. glabrata*, *in vitro* cellular internalization, localization and intracellular trafficking were explored by TEM. **Figure 5-8** and **Figure 5-9** represent the TEM images for *C. albicans* and *C. glabrata* clinical isolates, respectively, which were incubated with AMB-IONP for 4 hours. IONP are easily traceable with TEM imaging. TEM images revealed the nanoparticles were localized at or near the cell wall and cell membrane and inside the cytoplasm, nucleus and endolysosomal vesicles for both isolates tested.

Through TEM images one can also derive additional information related to the actions of a delivery system on the cells upon internalization. After 4 hours of treatment, the TEM images demonstrated the disorganization of the cytoplasm with deformed nuclei and other intracellular components including endoplasmic reticulum and mitochondria (**Figure 5-8** and **Figure 5-9**). Also observed was a significant increase in the number of

Figure 5-7. Uptake mechanism study with TEM for *C. glabrata* treated with AMB-IONP

The uptake mechanism was observed with TEM and the image of *C. glabrata* cells incubated with AMB-IONP for 4 hours revealed clathrin-mediated endocytosis, caveolar-type endocytosis, and lipid-raft-mediated endocytosis as major uptake pathways.

Figure 5-8. Intracellular trafficking localization of AMB-IONP in *C. albicans* by TEM

TEM images revealed that the nanoparticles were localized at or near the cell wall and membrane and inside the cytoplasm, nucleus and endolysosomal vesicles, as indicated by arrows in images 'a' through 'f'. The image 'a' indicates a dying cell with disorganization of intracellular components.

Figure 5-9. Intracellular trafficking localization of AMB-IONP in *C. glabrata* by TEM

TEM images revealed the nanoparticles were localized at or near the cell wall and membrane and inside the cytoplasm, nucleus and endolysosomal vesicles, as indicated by arrows in images a through e. The image 'f' indicates a dying cell with disorganization of intracellular components.

vacuoles [290]. The increase in vacuole number could be correlated to the actions of AMB that extracts ergosterol. Toxic ergosterol precursors could possibly accumulate in the vacuoles. The images also revealed the disintegration of the cell wall and cytoplasmic membrane along with cytoplasm leakage. These findings indicate dying cells. See **Figure 5-8d** and **Figure 5-9f**.

Confocal fluorescence provided evidence of colocalization of fluorescence signals from nuclei (blue), endosomes and lysosomes (deep red), and fluorescent labeled AMB-IONP (green). After 4 hours of incubation of clinical isolates of *C. albicans* and *C. glabrata* with the nanoparticles, **Figure 5-10** shows colocalization, which are in agreement and compliment the results of the TEM images.

5.3. Conclusions

Studies conducted to investigate the *in vitro* cellular association, cellular uptake mechanisms, and intracellular trafficking were successfully accomplished with clinical isolates of *C. albicans* and *C. glabrata*. These isolates were treated with AMB-IONP and for intracellular confocal imaging, the particles were labelled with Coumarin 6 dye. The targeting capability of AMB-IONP was evidenced by the cell association experiments. This study revealed that the uptake mechanism was energy dependent as reduced uptake of particles was observed at the lower temperature (4°C). The endocytosis inhibitor evaluation and TEM images revealed the major particle uptake pathway for *C. albicans* was lipid-raft mediated endocytosis. Whereas for *C. glabrata* clinical isolate, the major uptake mechanisms were multiple and included clathrin-mediated endocytosis, caveolar-type endocytosis, and lipid-raft-mediated endocytosis.

The intracellular trafficking of the AMB-IONP were observed with the help of TEM and confocal laser scanning microscopy. TEM images provided the evidence that the AMB-IONP were localized at or near the cell wall and membrane and inside the cytoplasm, nucleus and endolysosomal vesicles for both isolates tested. The confocal study confirmed the colocalization of fluorescence signals from nuclei, endosomes and lysosomes, and fluorescent labeled AMB-IONP, four hours after treatment.

In summary, these results suggest that AMB-IONP targeted drug delivery system was successfully delivering AMB payload at and/ inside the fungal cells leading to potential therapeutic advantages over AMB-D.

Figure 5-10. Intracellular trafficking localization of AMB-IONP in *C. albicans* and *C. glabrata* by confocal microscopy

The confocal fluorescence revealed the colocalization of fluorescence signals from nucleus (blue), endosomes and lysosomes (deep red), and fluorescent labeled AMB-IONP (green), after 4 hours of incubation of clinical isolates with nanoparticles.

CHAPTER 6. LYOPHILIZATION AND SHORT TERM STABILITY OF AMB-IONP

All drug products eventually degrade and make them unacceptable for human use. Knowing the drug product's stability limits help assure the quality of the product during testing, manufacturing and clinical trials. Some products may only be stable for an hour or less while others may have a shelf life of years. For instance, during clinical trials, some chronic pain studies have depended on repeated extemporaneous compounding of capsaicin solution to produce test dosage forms for clinical trials [291]. However, more reproducible dosage forms were produced once the long term stability profile was established [291]. This means fewer batches will be made and less batch to batch variation will exist for the drug product during clinical trials. Additionally, establishing and knowing the stability characteristics of a drug product are a mandatory step in reproducing a quality drug product acceptable for human testing.

For drug products to be stable and produce their maximum effectiveness both the chemical properties such as active drug concentration and physical properties such as pH, color and clarity of solution products must be within the specification of the critical quality parameters set for the product during the development and testing phases. For example, the required active drug potency must be above approximately 90% of the label concentration or greater [292]. Physical property changes such as color changes or precipitate in a true solution product with controlled storage are unacceptable. Since the rate of degradation relates to temperature, sometimes relative humidity and other storage conditions, stability studies are mandatory to produce a FDA registered product. The guidelines to produce the required scientific information are found in the ICH international guidelines on drug stability investigations [293].

In general, true solution injectable drug products are the most common and easiest to produce. However, as formulation complexity increases, more parameters are needed. For example, multiphasic systems such as emulsions, suspensions, microemulsions and micellar solutions require more precise parameters to establish the physical stability of the more complex products. Nanoparticle containing medicines need to be stored and tested under various temperatures with time, but there must be specifications for the particle size average, particle size distribution, particle surface charge and drug potency [28]. Additionally, nanomedicine particles should not usually aggregate unacceptably under general storage conditions since the product usually continues to degrade. Therefore, products that are more complex than true solution salts of drug molecules have a tendency to be more labile. This often means these products require special processing, storage and testing. With nanomedicine particle dispersions in liquids, lyophilization is often needed to produce a reasonable product shelf life of two years or greater.

Over the last twenty to thirty years pharmaceutical lyophilization has become a common method to extend delicate product shelf life [294]. Lyophilization is essentially the process of eliminating water from a frozen product with time, temperature controls and vacuum by a machine controlled method. Lyophilization requires special equipment,

facilities and personnel training to operate a lyophilization manufacturing process. To use a lyophilized injection, usually sterile water or a buffer solution are used to reconstitute the dried drug powder back into a liquid. Therefore, the stability of both the stored dried product and the reconstituted liquid product are studied.

The specific aim was to develop a lyophilized AMB-IONP product to improve the shelf-life of the formulation during storage for long term usage. Sucrose was studied as its lyoprotective ability at various weight ratios to develop an acceptable lyophilized product. Finally, the short term stability of the liquid dispersion and lyophilized product of AMB-IONP (design D) were evaluated at different storage conditions.

6.1. Materials and Methods

6.1.1. Materials

Sucrose was purchased from Sigma-Aldrich (St. Louis, MI, USA). T-Zero aluminum pans were bought from TA Instruments (New Castle, DE, USA). The oleic acid coated IONP, activation buffer, coupling buffer, quenching buffer, wash/storage buffer, and SuperMag Separator™ magnetic separator were obtained from Ocean NanoTech (Springdale, AR, USA). EDAC was purchased from Acros (Pittsburg, PA, USA) and NHS was purchased from Thermo Scientific (Rockford, IL, USA). BSA (Cohn fraction V, lyophilized powder, MW~66000, purity 95-99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). AMB USP analytical standard was obtained from MP Biomedicals (Santa Ana, CA, USA) and HPLC grade methanol, acetonitrile, DMSO, acetic acid, water, and 2 mL tubular vials were obtained from Fisher (Pittsburg, PA, USA). 13 mm lyophilization rubber stoppers and seals were obtained from West (Cincinnati, OH, USA).

6.1.2. Preparation of AMB-IONP

Formulation design D of AMB-IONP were prepared by the technique previously detailed in Chapter 3.

6.1.3. Determination of Tg'

Tg', the glass transition temperature of maximally freeze-concentrated amorphous phase of mixture of AMB-IONP and sucrose was determined by differential scanning calorimetry (DSC) with a Q2000 differential scanning calorimeter connected with cooling system (TA Instruments, New Castle, DE, USA). The sample mixture of 10-20 µL was placed in T-Zero aluminum pans and sealed hermetically. An empty pan was prepared similarly and used as reference. The samples were equilibrated at -60°C. After equilibration, the samples were heated at the rate of 5°C/minute to 20°C.

6.1.4. Lyophilization of AMB-IONP

AMB-IONP (design D) were prepared as detailed in Chapter 3 and a lyoprotectant sucrose was added to the formulation. Different weight ratios of AMB-IONP to sugar were used (1:0, 1:1, 1:4, 1:8, 1:16, and 1:20). The mixture of 500 μ L were filled into 2 mL glass lyophilization vials and rubber lyophilization stoppers were placed over the vials in such a fashion that the stoppers was partial opened to vent the moisture. The lyophilization vials with stoppers were placed into the lyophilization tray and loaded into the laboratory scale benchtop VirTis adVantage Plus lyophilizer (SP Scientific, Gardiner, NY, USA). The lyophilization cycle recipe was developed based on the Tg' of maximally freeze-concentrated amorphous phase, determined by DSC. The lyophilization cycle recipe consisted of a freezing shelf temperature of -35°C, primary drying shelf temperature of -30°C, and secondary drying shelf temperature of 15°C with primary and secondary drying chamber pressure of 100 mTorr. The lyophilizer was operated and the product temperature, shelf temperature, and condenser temperature were controlled and monitored using Wizard 2.0 software. Upon finishing the lyophilization the product in the vials were overlaid with nitrogen at atmospheric pressure, stoppered and sealed.

6.1.5. Visual inspection and physicochemical characterization of lyophilized AMB-IONP

The lyophilized product of the AMB-IONP was subjected to visual inspection to determine height of the cake, any cake separation, product on rubber closures, meltback, and presence of any surface abnormalities such as air bubbles or puffing.

The lyophilized cake was then subjected to moisture content analysis using a Mettler Toledo DL31 Karl Fischer Titrator (Mettler Toledo Inc., Boston, MA, USA). The equipment was calibrated before use on the day of analysis. The sample of about 5 mg of the lyophilized product was weighed accurately and added to the titration mixture. The measurements were performed in triplicates and the moisture content in the lyophilized cake was calculated as % w/w.

The lyophilized product was reconstituted with water and the time for re-dispersibility after reconstitution was recorded. The mean hydrodynamic size (Z-average) of reconstituted AMB-IONP were determined by dynamic light scattering technique using a Malvern Zetasizer Nano ZS90 (Westborough, MA, USA) 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 diluted with HPLC grade water for the determination of Z-average. Each sample was determined in replicate. ζ -potential was estimated with electrophoretic light scattering technique with the Malvern Zetasizer. Samples were diluted in HPLC grade water filled into the disposable capillary cell (DTS1070) for duplicate analysis.

6.1.6. Stability evaluation of AMB-IONP

The aqueous dispersion and lyophilized product of AMB-IONP (design D) was subjected to short term stability study for up to two months. Samples were randomly selected from the batch, protected from light and placed in temperature controlled environments under refrigerated (2-8°C) and room temperature conditions (25°C). Each vial was collected from each temperature controlled environment at predetermined time points of one, two, three, four, and ten weeks and subjected to physicochemical stability analysis. Furthermore, a short term (6-8 hours) stability test for the reconstituted vials at both temperatures conditions was performed. At each time point product in the vials were evaluated for presence of precipitate or any abnormal occurrences using a validated HPLC method. Potency testing at each sampling point and after 6-8 hours on reconstituted vials stored at refrigerated (2-8°C) and at room temperature (25°C) conditions was conducted using a validated HPLC method detailed in Chapter 2. For potency testing at each time point, the free drug was removed by washing with a wash/storage buffer and subjected to magnetic separation. In addition, the samples were also tested for particle size, PDI, and ζ -potential with the help of the Malvern Zetasizer as detailed in Chapter 3. Moisture content was also determined with Karl Fischer Titrator at predetermined time points of day 0 and end of ten weeks.

The physical characteristics of the dispersions including reconstituted product were evaluated qualitatively at each sampling point. Each sample was visually inspected without magnification for changes in color, clarity, particulate matter, and product/container closure abnormalities.

6.2. Results and Discussion

6.2.1. The Tg' determination for development of lyophilization cycle

The stability of AMB in dispersion form during storage is a major challenge in the development of IONP formulations. Physical instability leads to leakage of loaded drug and aggregation of particles resulting in increased particle size [28]. The physicochemical stability can be improved by removing water from the formulation by lyophilizing the product. Water tends to crystallize during the freezing step resulting in ice crystals, during which the nanoparticles and solute show a tendency for fusion and aggregation due to their higher concentration at this stage. To avoid this problem lyoprotectant sugar can be added. The sugar has a capability of forming an amorphous matrix around the particles that protect from fusion and prevent rupture of the particles due to crystallization of ice [34, 294]. Even though trehalose is the most widely used lyoprotectant, reports indicate that sucrose has better control over nanoparticles size than trehalose [28, 295]. Additionally, trehalose can also undergo crystallization during lyophilization. The integrity of the nanoparticles could be compromised due to crystallization during lyophilization. Thus, trehalose was not used and sucrose (non-crystallizing solute) was

evaluated as a lyoprotectant in an effort to development of lyophilized AMB-IONP formulation.

The T_g' of the maximally frozen concentrated mixture of the formulation recipe is an important factor in determining the freezing and primary drying temperatures when developing a lyophilization. In order to dry the formulation product without losing its structural properties, the formulation dispersion should be maintained in the solid state below the glass transition (collapse) temperature. T_g' was determined with the help of DSC using incremental weight ratios of IONP to sucrose as lyoprotectant. The DSC thermograms of different sucrose ratios to IONP were exhibited in **Figure 6-1**. The data indicates that the T_g' values increased with an increase of sucrose weight ratio in the mixture [296]. The T_g' value was found to be about -25°C . Thus, primary drying temperature was optimized to $2-5^{\circ}\text{C} < T_g'$ to inhibit collapse. The temperature of -35°C was found optimum for a freezing temperature during the lyophilization process with the cooling rate of $\sim 0.4^{\circ}\text{C}/\text{minute}$ and then held at this temperature for 6 hours. The primary drying temperature of -30°C was found to be optimum to avoid collapse of the cake. The end of primary drying cycle was determined by the increase in product temperature that matched with the shelf temperature of the lyophilizer. The total hold time of 12 hours at -30°C was applied. The chamber pressure of 100 mTorr was used, which is below the vapor pressure of ice at sub-zero temperature [297, 298]. The main reason for the sublimation process during the primary drying cycle was the difference in the ice vapor pressure at -30°C and the chamber pressure of the lyophilizer. The temperature of 15°C was used as the secondary drying temperature and held for 6 hours at that temperature with maximum vacuum of 100 mTorr. The product was then removed from the lyophilizer, overlaid with nitrogen and sealed.

6.2.2. Visual inspection and physicochemical characterization of lyophilized AMB-IONP

The lyophilized product vials were then subjected to visual inspection and for physicochemical characterization. The product with different sucrose weight ratios were visually inspected to determine the cake height, any cake separation, product on rubber closures, and presence of any surface abnormalities such as air bubbles or puffing. The lyophilized product with no sucrose exhibited an unacceptable meltback cake (**Figure 6-2**). The product with sucrose exhibited no cake separation, no product on rubber stoppers, and there was visible surface abnormalities. However, there was significant change in cake height to the initial fill volume when sucrose weight ratio of 8 or lower was used (**Figure 6-2**). The reduction in cake height is significant for poor and unacceptable cakes. The sucrose weight ratio of 16 and higher was found to be optimum without any visible abnormalities. In addition, the lyophilized product was found to be instantaneously redispersed upon reconstitution with water.

The mean size, PDI and zeta potential of AMB-IONP were found to be retained after lyophilization at 1:16 weight ratio of IONP to sucrose (**Table 6-1**). At lower sucrose ratios, the particle size slightly increased up to 5nm in diameter suggesting that fusion

Figure 6-1. DSC thermograms of different sucrose weight ratios to IONP

The data indicates that the T_g' values increased with an increase of sucrose weight ratio in the mixture. The T_g' value was found to be about -25°C . Thus, primary drying temperature of $2-5^{\circ}\text{C} < T_g'$ was used to inhibit collapse.

Figure 6-2. Physical appearance of lyophilized product of AMB-IONP with different weight ratios of sucrose

Sucrose weight ratio of 16 and higher found to be optimum without any visible abnormalities of the cake.

Table 6-1. Characterization of lyophilized AMB-IONP with different weights of sucrose

Parameter	Before lyo (control)	After lyo (sucrose weight ratio to the weight of IONP)				
		20	16	8	4	1
Size (nm)	33.79±1.02	35.01±0.12	34.49±0.07	36.40±0.69	38.97±0.21	36.88±0.04
PDI	0.202±0.014	0.160±0.016	0.141±0.006	0.203±0.013	0.256±0.015	0.216±0.005
ζ-potential (-mV)	22.3±5.4	33.9±0.9	25.1±1.5	13.3±4.7	31.3±0.9	35.1±2.2

The data were presented as mean±SD.

and aggregation of IONP occurred during lyophilization. Overall, although the physicochemical properties of the nanoparticles were retained at lower sucrose ratios, reduction of the cake height to initial fill volume was witnessed. One of the critical quality attribute for any lyophilized product is the amount of the residual moisture content. In general, higher moisture content potentially leads to poor stability with loss of therapeutic activity [299]. It is recommended to have lower residual moisture content of 0.5-3% w/w for lyophilized products containing both small molecular weight drugs and molecules with significantly higher molecular weights [300]. The Karl Fisher analysis revealed a moisture content of <1.5% w/w for the lyophilized product of AMB-IONP containing sucrose (1:16), which is considered optimum for long term storage and was within the acceptable limits set by the FDA [301]. Thus, sucrose weight ratio of 16 to IONP was selected as optimum for the lyophilization formulation based on visual inspection and physicochemical characterization.

6.2.3. Stability of aqueous dispersion and lyophilized AMB-IONP

The stability of the liquid dispersions and lyophilized formulations of AMB-IONP (design D) were evaluated in 2 cc glass vials (protected from light) at 2-8°C (refrigerated condition) and 25°C (room temperature condition) for up to two months. At each predetermined time point the physicochemical characters such as potency, mean particle size, PDI, ζ -potential were determined. The stability data for 2-8°C and 25°C were presented in **Figure 6-3**. The liquid dispersion stored at ambient conditions of 25°C showed significant degradation by the end of the second week of storage and was discontinued from the study. The lyophilized product stored at 2-8°C exhibited good stability with more than 90% of drug retained even after ten weeks. The next best stable product was the lyophilized product stored at 25°C with up to 94% of drug retained in ten weeks. The liquid dispersion product stored at 2-8°C did not exhibit good stability with only 8% drug retained by end of the study. These results indicate that AMB is sensitive to temperature and exhibit thermal degradation, which is in good agreement with a previous report by Manosroi et al. in 2004 [302]. Drug is more stable in dry form in lyophilized cakes rather than in aqueous medium. Removal of water from the liquid dispersion through lyophilization improved the shelf life by preventing drug degradation through hydrolysis or oxidation. In addition, the drug might be partitioning out from nanoparticles in an aqueous phase when compared to a lyophilized form. All the physicochemical characters of the AMB-IONP in both forms stored at both temperatures retained the mean particle size, PDI, ζ -potential without any significant changes and were within the acceptable limits (**Figure 6-4**). Visual inspection could not identify any physical abnormalities at each study time point. The moisture content of the lyophilized cake was maintained below 1.5% w/w throughout the testing period. The reconstituted lyophilized product was found to be stable at ambient conditions for up to 7 hours (data not presented). The lyophilized AMB-IONP might be the best formulation for AMB, due to its higher stability when stored at 2-8°C than liquid dispersion form and room temperature storage conditions.

Figure 6-3. Drug stability in lyophilized and liquid dispersion formulations

The data were presented as mean $\pm$ SD (n=3). The variability of data with time is attributed to the inconsistent amount of IONP loss during washing step for the removal of free drug.

Figure 6-4. Size, PDI, and ζ -potential stability in lyophilized and liquid dispersion formulations

The data were presented as mean $\pm$ SD. Graphs (a), (b), and (c) represents data for size, PDI, and ζ -potential, respectively. All the physicochemical characters of the AMB-IONP in both forms stored at both temperatures retained the mean particle size, PDI, ζ -potential without any significant changes and were within the acceptable limits.

6.3. Conclusions

The lyophilized formulation of AMB-IONP was successfully prepared with the selection of an appropriate amount of the lyoprotectant, sucrose. The thermal analysis of the lyophilized formulations with varied amounts of sucrose by DSC concluded with the optimum freezing temperature of -35°C for the lyophilization process as Tg' was found to be about -25°C. The primary drying temperature of -30°C was found to be optimum to avoid collapse of the cake. A sucrose weight ratio of 16 to the amount of IONP was determined to be ideal to retain the physicochemical properties of AMB-IONP after lyophilization with the desired cake height. The moisture content analyzed with Karl Fisher titration was found to be within 1.5 % w/w and is in good agreement with FDA guidelines [301].

The short term stability study of both formulations (lyophilized and aqueous dispersion) at 2-8°C (refrigerated condition) and 25°C (room temperature condition) for up to two months exhibited that the lyophilized form was more stable. Additionally better potency of AMB was observed with the product stored at refrigerated conditions.

CHAPTER 7. SUMMARY AND CONCLUSIONS

IFIs are associated with high morbidity and mortality, particularly in immunocompromised hosts who are at high risk for these infections. The mortality rates of some of IFIs go beyond 60 % in certain situations, and are one of the greatest challenges facing clinicians who care for immunocompromised patients. To date no ideal antifungal agent exists to cure IFIs. Current therapies with the most effective antifungal therapy, that includes AMB in different formulations, cause adverse effects such as nephrotoxicity and infusion related toxicity. Other problems such as pharmacokinetic variability, drug interactions and limited routes of administrations, limit its usage. Thus, a formulation with improved therapeutic efficacy and reduced adverse effects is needed by developing a targeted nanoparticle delivery system to the specific site of action, lowering the required dose and keeping AMB away from healthy cells. Additionally, it would be advantageous to integrate an imaging property within in the delivery system to follow the path of nanoparticles upon administration to diagnose and monitor the severity of the infection.

Nanomedicines are formulated as extraordinarily small particles. Usually nanomedicines are less than 200 nm in diameter. If the particles are a larger size they may not reach the intended site of action. If they are too small, the particles may be excreted in urine minimizing the total dose that will get to the infection. Here the nanoparticles are only about 30-40 nm nanospheres and are in the colloidal size. This range, size, shape and surface charge are determining factors as to whether or not a relatively stable liquid dispersion can be formulated for administration to the patient or presented for further dosage form development. Infected tissues become much more porous during acute inflammation and allow very small particles to enter the infection site sometimes resulting in areas displaying an EPR effect. The entrapped particles thus become small depots for an extended or sustained release at the infection sites.

For the formulations developed in this dissertation, the surface layer AMB acts as both an effective drug but also as a targeting agent for the nanoparticles. AMB is a naturally occurring surfactant and has a strong affinity for the ergosterol in the fungal membrane. This allows the particles to migrate to the infection sites and stay there as they slowly dissolve. The AMB binding ability greatly simplifies the particle design in that an additional targeting layer does not need to be added to the nanoparticles. With this design, the layering process and eventual small scale production is greatly simplified for animal studies and clinical trials. Therefore, it is expected that these processes and properties should be more amenable to production scale-up as larger batches will be needed for further product testing.

The overall objective of this dissertation was to design, develop, characterize and evaluate a targeted nanotheronostic drug delivery system to diagnose and treat life threatening IFIs such as cryptococcal meningitis. This approach offers a possibility of reduction in nonspecific toxicities by administering lower but more accurately targeted doses. Additionally these IONP will improve the magnetic contrast for MRI and be useful

as diagnostic/imaging agent. The central hypothesis was that an AMB-IONP could be designed and constructed that would be expected to improve the therapeutic efficacy of AMB while minimizing dose associated toxicities in the treatment of IFIs compared to AMB-D.

Therefore, the studies conducted here are intended to develop a nanotheranostic targeted drug delivery system for imaging and treatment purpose. To address these development processes, I used *in vitro* models of the fungal clinical isolates for my proof of concept. This contained four main objectives. First, formulate an IONP (35-50nm) containing AMB and coated with BSA/HSA, applying a layer by layer approach. Then, convert the above formulated system in to a targeted drug delivery system by targeting with AMB itself in low quantity (AMB-IONP). Second, evaluate the uptake and efficacy of formulated targeted delivery system by performing *in vitro* studies in fungal clinical isolates. Third, evaluate the cellular uptake mechanisms and intracellular trafficking for a formulated targeted delivery system by performing *in vitro* studies in selected fungal clinical isolates. Fourth, lyophilize and develop a stable AMB loaded targeted IONP (AMB-IONP) suitable for clinical trials.

A validated isocratic HPLC method was developed for the determination of AMB in human plasma samples. The method was simple, rapid, accurate, and precise. Additionally, relatively shorter retention times (<3 min per injection) of AMB allow the analysis of a large number of samples in a short period of time. Therefore, this rapid and inexpensive method should be suitable for the routine analysis of AMB in dosage forms, clinical settings and to evaluate its pharmacokinetic, bioequivalence, and toxicokinetic profile.

The formulation designs by changing the order of the layers of AMB and BSA in the development of AMB-IONP were identified and optimized. The different designs were developed to increase the drug loading capability, to have the AMB on the surface for a targeting effect and to minimize the processing steps. The approach was successful with design D being the most fruitful in that regard with maximum drug loading of $13.6 \pm 6.9 \mu\text{g}$ of AMB/mg of nanoparticle. The size and ζ -potential for all the formulation designs were found to be optimum for a nanomedicine with $\leq 36 \text{ nm}$ and $\sim -20 \text{ mV}$, respectively. The PDI was also optimum at ≤ 0.2 PDI. Changing the order of AMB and BSA did not significantly affect the physical characteristics of the formulation designs, which is also evidenced by TEM imaging. The TEM images confirmed that the nanoparticles before and after formulating the designs were monodispersed and spherical in shape.

The drug release profile indicated a burst release up to 3 hours for design A and B, followed by a sustained drug release profile up to at least 72 hours. The best potential designs, C and D, indicated a sustain drug release profile up to 72 hours. Design D even in the presence of 4% albumin as a dissolution medium exhibited a sustained drug release profile with and without a glutaraldehyde cross linker. The major mechanism of drug release from the formulations was found to be Fickian and non-Fickian diffusion with first order and Higuchi kinetic models as best fit. The design D formulation was stable in

50% serum for up to 24 hours.

In vitro experiments for the determination of cellular uptake and the susceptibility testing were conducted. The flow cytometry results for cellular uptake of formulation design D in *C. albicans* and *C. glabrata* clinical isolates exhibited time dependent uptake. The cellular uptake reached a plateau between 0.5 and 1 hour and between 2 and 4 hours for *C. albicans* and *C. glabrata*, respectively. These results were in good agreement with confocal scanning laser microscopy images. All the formulation designs of AMB-IONP exhibited improved efficacy over marketed AMB-D product in the susceptibility testing conducted in clinical isolates of *C. albicans* SC5314, *C. glabrata* 66032, *C. krusei* CK1, *C. parapsilosis* CP1, and *C. tropicalis* CT1. Design D was found to be superior and was 16-25 fold more efficacious than the conventional product (AMB-D). Even in clinical isolates of *C. albicans* CA1008 and Calowa60 that has < 1% of ergosterol, design D had better killing power over AMB-D. The initial studies with transferrin as a targeting ligand bound to the surface of design D formulation also exhibited improved efficacy over AMB-D for isolates tested. Therefore, transferrin could be an alternative targeting ligand, particularly for fungal meningitis, which is overexpressed with transferrin receptors during infection. Additionally, this formulation design could also be used to load multiple drugs that have different binding regions on BSA or HSA such as AMB and curcumin.

Studies conducted to investigate the *in vitro* cellular association, cellular uptake mechanisms, and intracellular trafficking were successfully accomplished with clinical isolates of *C. albicans* and *C. glabrata*. These isolates were treated with AMB-IONP and for intracellular confocal imaging, the particles were labelled with coumarin 6 dye. The targeting capability of AMB-IONP was evidenced by the cell association experiments. This revealed the uptake mechanism was energy dependent as reduced uptake of particles was observed at a lower temperature (4°C). The endocytosis inhibitor evaluation and TEM images revealed the major particle uptake pathway for *C. albicans* were lipid-raft mediated endocytosis. Whereas for *C. glabrata* clinical isolate, the major uptake mechanisms were multiple and included clathrin-mediated endocytosis, caveolar-type endocytosis, and lipid-raft-mediated endocytosis.

The intracellular trafficking of the AMB-IONP were observed with the help of TEM and confocal laser scanning microscopy. TEM images provided the evidence that the AMB-IONP were localized at or near the cell wall and cell membrane and inside the cytoplasm, nucleus and endolysosomal vesicles for both isolates tested. The confocal study confirmed the colocalization of fluorescence signals from nuclei, endosomes and lysosomes, and fluorescent labeled AMB-IONP, four hours after treatment. These results suggested the AMB-IONP targeted drug delivery system was successfully delivering AMB payload at and/ inside the fungal cells leading to potential therapeutic advantages over AMB-D.

The lyophilized formulation of AMB-IONP were successfully prepared with the selection of appropriate amount of the lyoprotectant, sucrose. The thermal analysis of these lyophilized formulations with varied amounts of sucrose by DSC concluded with the optimum freezing temperature of -35°C for the lyophilization process as Tg' was found to

be about -25°C. The primary drying temperature of -30°C was found to be optimum to avoid collapse of the cake. A sucrose weight ratio of 16 to the amount of IONP was determined to be ideal to retain the physicochemical properties of AMB-IONP after lyophilization with the desired cake height. The moisture content analyzed with Karl Fisher titration was found to be within 1.5% w/w and was in good agreement with FDA guidelines.

A short term stability study of both formulations (lyophilized and aqueous dispersion) at 2-8°C (refrigerated condition) and 25°C (room temperature condition) for up to two months exhibited that the lyophilized form is stable. Additionally better potency of AMB was observed with the product stored at refrigerated conditions.

A targeted nanotheranostic drug delivery system (AMB-IONP) was successfully designed, developed, characterized and evaluated. Therefore, I accept the central hypothesis of my dissertation that I could develop a potential drug product for IFI treatment. The unique nature of this parenteral formulation has the potential to overcome the barriers in chemotherapy of drugs for deadly fungal infections. The information generated from these studies may potentially cure IFIs with better therapeutic efficacy and minimum toxicity, when compared to the current therapy. The degree of change in the patient's quality of life and eventual life expectancy is expected to improve by the targeting ability of the antifungal treatment. In the future, I would like to evaluate AMB-IONP theranostic properties in animal models to diagnose and treat IFIs. This will also allow me to characterize the dosage form and to advance the understanding of drug release from these formulations in mammalian systems.

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VITA

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The scientific and engineering training of Pavan in the areas of analytical methodology and nanomedicine product development have enabled him to perform and publish in a wide range of high impact scientific journals. He has been invited to share his scientific findings at workshops and professional conferences in the United States, including meetings hosted by the American Association of Pharmaceutical Scientists (AAPS). His scientific credibility enabled him to serve as an editorial board member and reviewer for several international journals. He has been elected for membership in several pharmaceutical and biomedical professional societies, including American Association for Cancer Research, Sigma Xi, AAPS, American Chemical Society, American Society for Microbiology, the Rho Chi Society, and the Parenteral Drug Association. He was elected as an *AAPS Student Chapter Vice Chair* at UTHSC (2014-2015). Pavan was recipient of the following distinctions: Employee Service Award (2014), UTHSC; Oral Presenter Award (2014), Drug Discovery and Development Colloquium, Little Rock, Arkansas; Outstanding Performance for AAPS Student Chapter Vice Chair at UTHSC (2014-2015); College of Graduate Health Sciences Travel Award (2015), UTHSC; and American Society for Microbiology Student and Post Doctoral Fellows Travel Grant (2015), Interscience Conference of Antimicrobial Agents and Chemotherapy (ICAAC)/International Congress of Chemotherapy and Infection (ICC).