Pharmaceutical Studies of Amphotericin-B:

Oral Formulation using Nanotechnology

A thesis submitted for the degree of Doctor of Philosophy by

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January 2016

Abstract

Amphotericin B (AmB) is the gold standard treatment of systemic fungal infections. It is the secondary treatment of leishmaniasis with 97% cure rate with no reported resistance. However, the only dosage form of AmB in the market is parenteral formulations as intermittent iv infusion. Sodium deoxycholate (Fungizone®) was the first conventional formulation followed by several new lipid formulations since 1990 (Ablecet®, Amphocil® and Ambisone®). These formulations were proved to be very effective, less toxic and require shorter course of therapy compared to Fungizone®. Unfortunately, all these parenteral formulations suffer from the inconvenience of intravenous (iv) administration, required hospitalization, more importantly, induce a number of side effects, and are very expensive.

Therefore, the need for oral formulations of AmB is a crucial concern. During the last four decades there have been several published efforts representing different strategies in formulating oral delivery system of AmB with reduced toxicity. None of these formulations has been introduced into the market yet.

In this study a novel stealth nanotechnology formulations containing AmB have been developed for an oral administration in order to improve its solubility, bioavailability an with lower toxicity. The selection of the copolymer used PEGylated poly-lactic- co-glycolic acid (PLGA-PEG) was based on the hypothesis that FDA has approved each individual component of the copolymers for oral and parenteral administrations of poorly soluble drugs.

The development of these oral formulations of AmB went through several optimization steps, including method of preparation, polymer selection, stirring rate, cosolvency, pH, particle size reduction, stabilizers, surfactants, absorption enhancer, and HPLC assay for *in vitro* analysis.

Novel oral biodegradable stealth polymeric nanoparticles have been successfully fabricated using the commercially available PLGA-PEG copolymer for AmB by emulsion diffusion method. *In vitro* characterizations of the lyophilized formulations showed that

main particles size (MPS) of AmB loaded to NPs ranged from 23.8 ±4.8 to 1068± 489.8 nm. An increased stirring rate favored AmB NPs with smaller MPS. There was a significant reduction in MPS ($P < 0.05$) and significant increment in the drug content ($P < 0.05$) and the amount of drug released ($P < 0.05$), when AmB-NPs were prepared using the diblock copolymer PLGA–PEG with 15% PEG compared to other copolymers investigated. The addition of three emulsifying agents poly vinyl pyrrolidone (PVP), Vitamin E (TPGS) and pluronic F-68 to AmB formulations led to significant reduction in particle size and increase in drug entrapment efficiency (DEE) compared to addition of PVP alone. Fourier transform infrared spectroscopy demonstrated a successful loading of AmB to stealth PLGA– PEG copolymers. PLGA–PEG copolymer entrapment efficiency of AmB was increased up to 56.7%, with 92.7% drug yield.

The physicochemical characterization done by SEM and TEM images indicate spherical particle in nanometer size in isolated and agglomerated state. The FTIR changes obtained from different AmB-NPs formulations suggesting some sort of interaction between the drug and the polymer.

A simple, fast, accurate and reliable HPLC method for the *in vitro* investigations (drug content and drug release from the NPs) of AmB has been developed. Intra-day and inter-day variability were $\leq 6.2\%$ with excellent linearity(R^2=0.9982) and reproducibility.

All tested formulations have shown slow initial release with 20% to 54% released within 24 h in phosphate buffer containing 2% sodium deoxycholate. The release rates of AmB from these formulations were best fit to the Korsmeyer–Peppas model. Therefore, a diffusion release model is the best to characterize the *in vitro* release of AmB from these formulations.

A new selective, sensitive and precise LC MS/MS method was developed to measure AmB concentrations via electrospray ionization source with positive ionization mode. The precision and accuracy of the developed LC MS/MS method in the concentration range of 100–4000 ng/ml show no significant difference among inter- and-intra-day analysis ($p > 0.05$). Linearity was observed over the investigated range with correlation coefficient, $R^2 > 0.9943$ (n = 6/day). The assay was able to detect all the drug

concentrations for pharmacokinetics and bioavailability estimation after oral dosing of AmB loaded to PLGA-PEG compared to its parenteral administration in rats. AmB solutions or NPs were kept in refrigerator during the study.

After PO administration AmB loaded to PLGA-PEG (C6), AmB was rapidly absorbed, distributed, and then slowly eliminated. The effect of glycyrrhizic acid (GA) as a natural absorption enhancer added during formulation or before administration was investigated. The addition of GA significantly ($P<0.05$) increased AmB AUC and C_{max} (2 - 3 folds). There was no significant change ($P=0.09$) in adding GA during formulation or before administration. A tremendous improvement of AmB oral absorption in rats by > 790 % than Fungizone® is observed after GA Addition.

AmB formulations have been screened for toxicity using several *in vitro* assays*;* hemolysis, antifungal activity and analysis of the cell viability by 3-(4,5dimethylthiazoly)-2,5-diphenyl-tetrazolium bromide(MTT) assay. Fungizone® was used as the reference standard. AmB loaded to PLGA-PEG of the selected formulations showed a significant reduction ($P< 0.05$) in the *in vitro* hemolytic activity as compared to Fungizone®. On cellular level, AmB loaded to PLGA-PEG showed a potent activity against the *in vitroC. albicans* with a parallel decrease in the cytotoxicity against in the *in vitro* tested cell lines. Additionally, no pathological abnormalities were observed in rats given iv administration of AmB protocol except after Fungizone® administration.

The *in vivo* nephrotoxicity and histopathological evaluation of AmB were performed in both rats' kidneys and liver using forty eight rats divided into eight groups after single and multiple iv administrations of 1mg/kg of the selected formulations or Fungizone® for a week. AmB induced nephrotoxicity was also estimated in these rats by measuring blood urea nitrogen and plasma creatinine (biomedical parameters). A significant ($P<0.05$) nephrotoxicity was observed after Fungizone® administration compared to all AmB loaded to PLGA-PEG formulations. The histopathological study of the isolated kidney tissues confirmed this finding. Furthermore, the incidence of the liver toxicity due to the selected AmB loaded PLGA-PEG formulations has been reduced significantly as investigated through *in vivo* analysis (histopathological and biochemical tests) in comparison with Fungizone®.

In conclusion, a successful novel AmB oral formulation with improved efficacy and bioavailability and with less toxicity to the kidney and the liver was developed. Further investigations are needed in the near future for this formulation to be in the market as an oral delivery system of AmB.

Dddication

To the sole of my parent who always encouraged me to be the best, I could.

To my beloved husband without him, I could not complete this work.

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Declaration

This dissertation is the outcome of my own work, except where references are made to other sources. The work of this thesis to get my Ph.D. has not been submitted to any other University or Institute to get any other degree or qualification.

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Acknowledgments

In the name of Almighty Allah most compassionate most merciful. Praise is to Almighty Allah and Peace is upon the Holy Prophet Mohamed and his Followers.

First, and foremost, I am deeply grateful to Almighty Allah for helping me through difficult times, guiding me through all my life and for giving me the strength, capability and opportunity to do this work so that today I can stand proud with my head held high.

There are no proper words to convey my deep gratitude and respect for my thesis and research advisor, Professor Mahasen Ali Radwan. This thesis would not have been possible without the help, support and extreme patient of her. I would like to express my deepest gratitude to her for her concern, guidance, and day to day encouragement whenever I needed and undeterred support and advice. I will always be grateful to you doctor for allowing me to be your student. I was very proud to work with you.

I am greatly indebted to my thesis external supervisor; Dr Lidija Siller for the time & effort she have allocated for me, her valuable guidance, advice and support that guided me through all my work, theoretical & practical, to finally reach the success.

I extend my sincere word of thanks to co-advisors; Professor Matthew C. Wright, Dr. Benjamin Horrocks and Dr. Elaine Mutch who have been always there, as teachers, and as scientists.

My sincere appreciation to my colleagues in the department of pharmaceutical department, pharmacy college King Saud University, Saudi Arabia for their kind support, and cooperation. Finally I would like to acknowledge the people who mean world to me, my family. For you, words will never be enough.

My parent, and close family, thanks you for being such wonderful persons and for giving me the drive to succeed and persevere. I admire you greatly for your

strength and unselfishness. I always get relieved by talking to you, and I am thankful to have your protection and love. I only hope I make you proud.

My husband and my best life partner, Qais, thank you for being such a wonderful friend and companion. My heartfelt gratitude for all your love, endless support, timely advices, incredible patient and consideration shown to me throughout the course of this work.

I love you all and I hope to make you always proud of me.

Published Papers and Conferences

Parts of the theses have been published in ISI Scientific journal:

1- Bushra Al-Quadeib, Mahasen A. Radwan, Lidija Siller, Benjamin Horrocks, Matthew C. Wright and Abdulaziz Alshaer (2014), 'Therapeutic monitoring of amphotericin B in Saudi ICU patients using UPLC MS/MS assay, Biomedical Chromatography Volume 28, Issue 12, 1652–1659.

2- Bushra T. AL-Quadeib, Mahasen A. Radwan, Lidija Siller, Benjamin Horrocks, and Matthew C. Wright (2015). Stealth Amphotericin B nanoparticles for oral drug delivery: *In vitro* optimization. Saudi Pharmaceutical Journal volume 23,290-302.

Parts of the theses have been presented in local and international conferences

1- Poster presentation**;** T3227: Therapeutic Monitoring of Amphotericin B in Saudi ICU Patients Using UPLC MS/MS Assay - See more at: http://app.imswift.com/aaps_2013/sessions/aaps13_T3227. AAPS Annual Meeting and Exposition.San Antino USA. November 12-15, 2013

2- Oral presentation**;** Therapeutic Monitoring of Amphotericin B in Saudi ICU Patients Using UPLC MS/MS Assay. First Annual international conference on Pharma 2013, Pharmaceutical Science and Pharmacology, 18-19 November 2013 Singapore.

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List of Abbreviations

ABCs	Amphiphilic block copolymer
ACN	Acetone
AE	Adverse effects
AmB	Amphotericin B
AmB-NP	Amphotericin B – Nanoparticle
ANOVA	Analysis of variance
AUC	Area under the curve
BCS	Biopharmaceutical classification system
BUN	Blood urea nitrogen
C	Carbon atom
^{0}C	Celsius degree
CNTs	Carbon nanotubes
C.V.	Coefficient of variation
DA	Dalton
DCM	Dichloromethane
DEE	Drug Entrapment Efficiency
DMSO	Dimethyl sulfoxide
DSC	Differential scanning calorimetry
FDA	Food and Drug Administration
ESD	Emulsification diffusion method
FTIR	Fourier transform infrared spectroscopy
IS	Internal standard
iv	Intra venous

ivi	Intra venous infusion
GA	Glycyrrhizic acid
GI	Gastrointestinal
GIT	Gastrointestinal tract
HCl	Hydrochloric acid
HLB	Hydrophilic lipophilic balance
HPLC	High performance liquid chromatography
KBr	Potassium Bromide
mg	Milligram
µg	microgram
µm	micrometer
MIC	Minimum inhibitory concentration
min	Minutes
ml	Milliliter
µl	Microliter
MPS	Mean particle size
ng	nanogram
N	Normal
nm	nanometer
NP	Nanoparticle
PCr	Plasma creatinine
PDI	Poly dispersity Index

PEG	Polyethylene glycol
PG	Propylene glycol
PGA	Poly Glycolic acid
PLGA	Poly lactic-co-glycolide copolymer
PKa	acid dissociation constant
PNPs	Polymeric nanoparticles
PO	Peroral
PVA	Polyvinyl alcohol
PVP	Polyvinyl pyrrolidone
QD	Quantum dots
R^2	coefficient of determination
RE	Relative error
RES	Reticuloendothelial system
RPM	Round per minute
RSD	Relative standard deviation
SEM	Scanning Electron Microscopy
SLN	Solid lipid nanoparticles
TCA	Tricyclic acid cycle
TEM	Transmission Electron Microscopy
TPGS	Vitamin –E derivatives
UPLC	Ultra-Performance Liquid Chromatography
US FDA	United State Food and Drug Administration
UK	United Kingdom
USA	United State of America
UV	Ultra violet
v/v	Volume per volume

w/w	Weight per weight
ZP	Zeta potential
ZS	Zeta sizer

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Chapter I. General Introduction

1.1. Nanotechnology and Nanoparticles in Drug Delivery Systems

A drug delivery system can be defined as macromolecules assemblies of suitable carrier in which a drug could be loaded to improve its efficacy. The development of this system for a drug is essential to improve the *in vivo* performance of its conventional dosage form concerning; poor water solubility, low bioavailability, instability, high toxicity, increased resistance or lower cellular uptake. This system is intended to deliver the drug mainly to the site of action to enhance its activity with minimal toxicity(Muhamad et al., 2012).

While, nanotechnology (particle size reduction technology) is the science of small; the very small to nanometers scale; 1.0 nanometer = 10^{-9} meter, nano is a billionth of a meter. When reducing the particle size, atoms and molecules behave differently than before reduction (Ochekpe et al., 2009, Pehlivan, 2013). The reduction in size significantly increases the particles' surface area leading to increase the solubility (Bawa, 2008).

A large variety of subjects benefit from the advantages of nanotechnology, such as electronics, healthcare, pharmaceuticals and food processing, etc.(Ochekpe et al., 2009, Alok et al., 2013).

Application of nanotechnology in pharmaceuticals focuses mainly on formulating therapeutic agents in biocompatible nanocarriers to be able to provide superior drug delivery systems for better management and treatment of diseases. These nanocarriers include polymeric nanoparticle, liposomes, solid lipid nanoparticles, nanoemulsions, carbon nanotubes, dendrimers, quantum dots and polymeric micelle (Sahoo et al., 2002, Mohanraj and Chen, 2007, Ochekpe et al., 2009, Zhang et al., 2010a, Kumari et al., 2010, Parveen et al., 2012).

Employing nanotechnology in drug delivery system is of utmost importance due to its potential to overcome a large number of challenges concerning not only the design of new drugs delivery systems, but also for the *in vivo* optimization of old drugs. Some of the drawbacks encountered by pharmaceutical development of most drug delivery systems are their poor drug solubility, intestinal absorption, bioavailability, *in vivo* stability, sustained

and targeted delivery to site of action, therapeutic effectiveness, side effects, and plasma fluctuations of drug concentration (Ochekpe et al., 2009).

Polymeric nanoparticle (PNPs) as shown in Figure 1.1, (Radwan and Aboul-Enein, 2002) as drug delivery system has been extensively investigated due to their targeting potential to the wanted site of action (Allémann et al., 1993, Bawa, 2008). PNPs can be defined as "solid particles with a size of 10-1000 nm". This tiny size range leads to a significant increase in the drug surface area and improved efficacy(Ranghar et al., 2014). PNPs can be classified according to the shape as spherical shapes, tubules, particles and tree like branched shapes. According to the method of preparation, the drug may be entrapped, dissolved, dispersed, encapsulated or attached to the nanoparticle matrix (Soppimath et al., 2001).

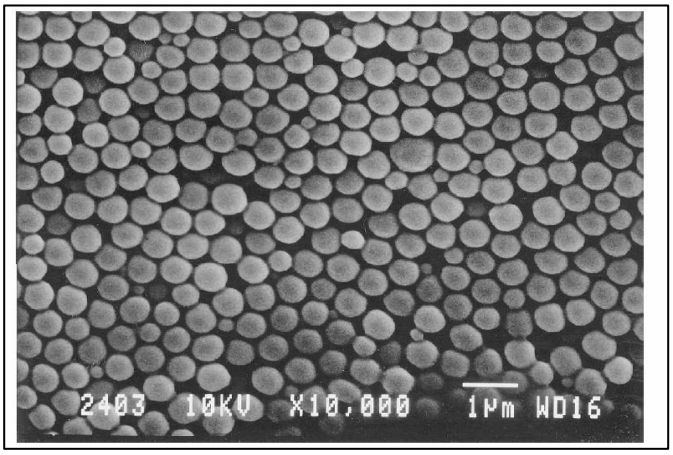


Figure 1.1. Scanning electron microscope for insulin-loaded polyethylcyanoacrylate nanoparticles(Radwan and Aboul-Enein, 2002)

Liposomes were first developed about 40 years ago (Martin, 1990). They are small artificial vesicles (50 – 200nm) (Figure 1.2(Xu et al., 2013)) composed of a lipid bi-layer membrane, similar to a cell membrane. Liposome can be composed of naturally derived phospholipids with mixed lipid chains (like egg phosphatidylethanolamine), or pure surfactant like dioleoylphosphatidyl-ethanolamine. They are used in biology, biochemistry, medicine, food and cosmetics. Due to its unique structure, liposome can encapsulate both

hydrophilic and hydrophobic molecules(Martin, 1990, Vemuri and Rhodes, 1995, Lemke et al., 2005, Ochekpe et al., 2009).

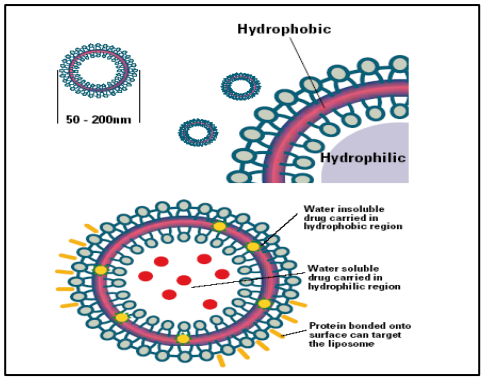


Figure 1.2. Basic component of liposome (Xu et al., 2013)

Solid lipid nanoparticles (SLNs) is shown in Figure 1.3(Uchechi et al., 2014),is typically spherical with an average diameter 10-1000 nm. SLN possess a solid lipid core matrix that can solubilize lipophilic molecules. These solid lipids are well tolerated by the body; however, SLN are suffering from insufficient drug loading, and relative high water content of the dispersions. SLN can be applied for parenteral, dermal, ocular, oral, pulmonary and rectal routes of administration (Schäfer-Korting et al., 1989, MuÈller et al., 2000, Müller et al., 2002, Hafner et al., 2014, Uchechi et al., 2014).

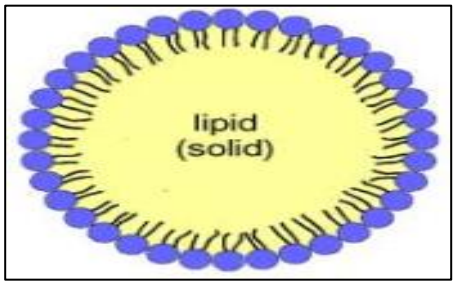


Figure 1.3. Basic component of solid lipid nanoparticles (Uchechi et al., 2014)

Nanoemulsions as shown in Figure 1.4(Uchechi et al., 2014)are emulsions with droplet size below 1.0 micron (1 micron= 1000 nm) in diameter. Unlike,

microemulsions(white in color) nanoemulsion are transparent. Nanoemulsions are biocompatible, biodegradable, reproducible and mainly used as a carrier for lipophilic drugs, Nanoemulsion can be applied for parenteral, oral, ocular, pulmonary and dermal deliveries(Ganta et al., 2010, Hafner et al., 2014, Caldeira et al., 2015).

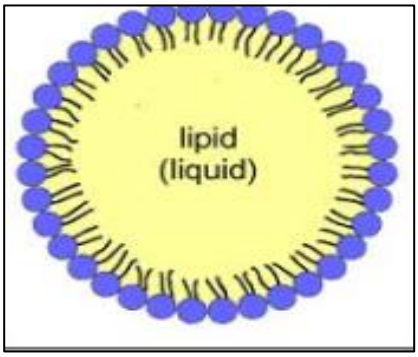


Figure 1.4. Basic components for nanoemulsion(Uchechi et al., 2014).

Carbone nanotubes (CNTs),as seen in Figure 1.5,were defined by (Pehlivan, 2013)as allotropes of carbon, made of graphite and built in cylindrical tubes with nanometer in diameter and several millimeters in length. CNTs have been successfully applied as therapeutic and diagnostic agents (drugs, genes, vaccines, antibodies, biosensors, etc.). They are considered as non-biodegradable drug targeting carrier.

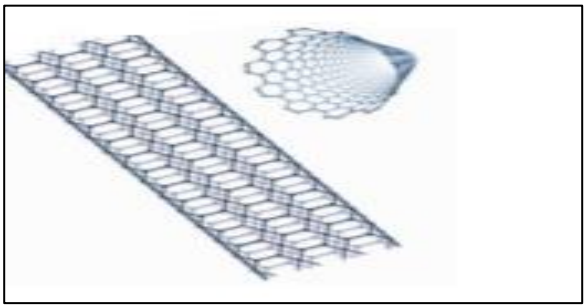


Figure 1.5. Carbone nanotube (Pehlivan, 2013)

Dendrimers (Figure 1.6)(Lee et al., 2005) are defined as synthetic nano-size tree like macromolecules with branching emanating from a central core “branched NPs”. Their distinctive structures allow drugs to be encapsulated within the central core as well as

attached on the surface. Both hydrophilic and hydrophobic drugs can be loaded to dendrimers (Hafner et al., 2014, Jain et al., 2015).

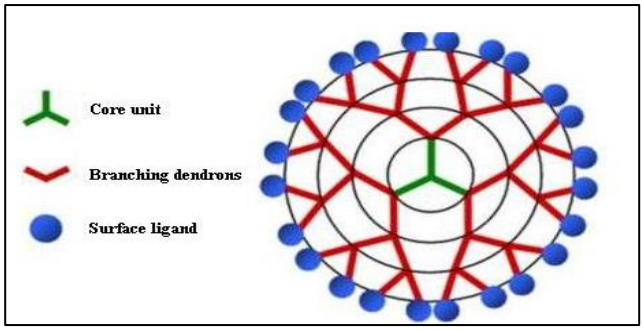


Figure 1.6. Basic dendrimers componentsLee et al. (2005)

Quantum dots (QDs) also called as fluorescence nanocrystals (Figure 1.7(Ghaderi et al., 2011) range from 2 to 10 nm in diameter. Structurally, some QDs possesses a bimetallic crystalline core, which is composed from materials including cadmium–selenium, cadmium– tellurium, indium–phosphate or indium–arsenate. A semiconductor shell typically zinc sulfide stabilizes the core, further improving the optical, physical properties, and bioavailability of the material (Ghaderi et al., 2011). QD shave increasing potential to be usedas replacements of organic dyes in imaging of biological systems as a result of their exceptional fluorescent properties, reasonable chemical stability and broad excitation ranges instead of organic dyes(Ghaderi et al., 2011).

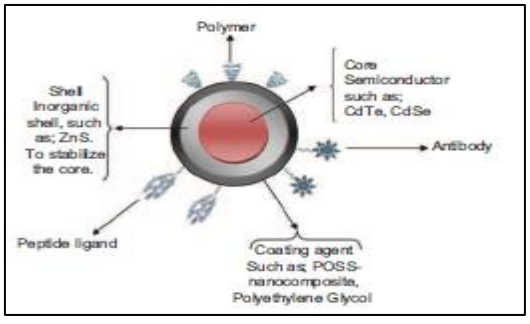


Figure 1.7. Structure of quantum dot with bioactive agents (Ghaderi et al., 2011)

Spherical polymeric micelles (Figure 1.8, (Xu et al., 2013) are nanoscopic core/shell structure formed by amphiphilic block copolymer. The inner core of a micelle is hydrophobic which, is bounded by a shell of hydrophilic polymers. Poorly water soluble and amphiphilic drugs can be encapsulated in the core, while the presence of hydrophilic shell extend their circulation time in the blood and improve their accumulation in cancer cells(Hafner et al., 2014).

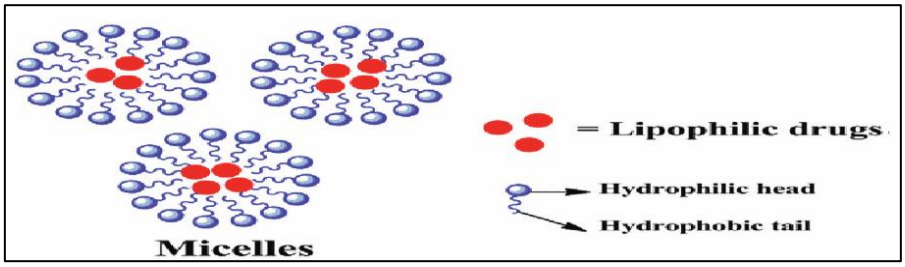


Figure 1.8. Polymeric micelles(Xu et al., 2013)

Over the last four decades, nanotechnology technique has been used extensively in the field of drug delivery systems resulting in a number of products, including therapeutics and imaging agents. Some of these nanomedicines were approved for the treatment of cancer, pain, and infectious diseases. Table 1.1 represents the commercially available nanotherapeutic drug delivery systems for oral and parenteral administrations (Murthy, 2007, Cho et al., 2008, Hafner et al., 2014). One of the major advantages that nanotechnology offers is targeted drug delivery to the site of action. Targeting can be categorized into three levels(Blanco et al., 2012) **(1**) delivery to a specific cancer region, i.e., central or peripheral, right or left; (2) delivery to the site of disease; and (3) delivery to distinct cell types with biological barrier transport, e.g., epithelial cells, or cells associated with lymphatic tissue. This can be achieved either through passive targeting of drugs to the site of action or by active targeting of the drug. Passive delivery refers to transport of the formulation through leaky tumor capillary fenestrations into the tumor interstitium and cells by passive diffusion or convection by utilizing specific carriers (Figure 1.9)(Blanco et al., 2012).

Table 1.1. **E**xamples of commercially available nanotherapeutic products* for oral and parenteral administration

Nanotechnology-based approach	Drug	Major indication	Dosage form	Route of administration	Brand name
Nanoemulsion	Sirolimus	Graft rejection, Kidney transplantation	Tablet	Oral	Rapamune®
	Aprepitant	Postoperative nausea and vomiting, Cancer	Capsule	Oral	Emend®
	Cyclosporine	Organ rejection	Soft capsules	Oral	Neoral®
	Olanzapine	Schizophrenia	Powder or suspension	iv/IM	Zypadhera®
Nanoparticles	Paclitaxel	Breast neoplasma	Powder or suspension	iv/IM	Abraxane®
Polymeric drugs	Sevelamer	Hyperphosphat-emia, Renal dialysis	Tablet	Oral	Renagel® Renvela®
	Albumin-Taxol	Anticancer	Solution	iv	Abraxane®
	PGA-Taxol	Anticancer	Solution	iv	Xyotax ®
	Glatiramer (copolymer)	Multiple sclerosis	Solution	SC	Copaxone®
Polymer-protein conjugates	Pegaspargase (mPEG-asparaginase)	Acute lymphobla-sticleukemia	Solution	iv/IM	Oncaspar®
	Certolizumab pegol(PEG-anti-TNFFab)	Rheumatoid arthritis	Solution	SC	Cimzia™
Liposomes	Amphotericin B	Fungal infections	Suspension	iv	AmBisome®
	Doxorubicin	Breast Neoplasms, Multiplemyeloma	Suspension	iv	Caelyx®
	Propofol	Anaesthetic	Emulsion	iv	Diprivan®
Nanocomplex	Sodium ferric gluconate	Iron deficiency anemia	Solution	iv	Ferrlecit®
	Ferric carboxymaltose	Iron deficiency anemia	Solution	iv	Ferinject®
Dendrimers		Vaginal microbicidal	Gel	SC	Viva®

*(Murthy, 2007, Cho et al., 2008, Hafner et al., 2014)

Carriers included in this targeting category are synthetic polymers, some natural polymers such as albumin, liposomes, micro (or nano) particles, and polymeric micelles(Muhamad et al., 2012). On the other hand, active targeting refers to increasing in the delivery of drugs to a specific target through the use of specific interactions at target sites where a drug's pharmacological activities are applied. These interactions for example include antigen–antibody and ligand–receptor binding. Alternatively, physical signals such as magnetic fields and temperatures that are externally applied to the target sites may be utilized for active targeting respectively. Carriers classified into this methodology include antibodies, transferrin, ferrite containing liposomes, peptides and thermoresponsive carriers (Blanco et al., 2012).

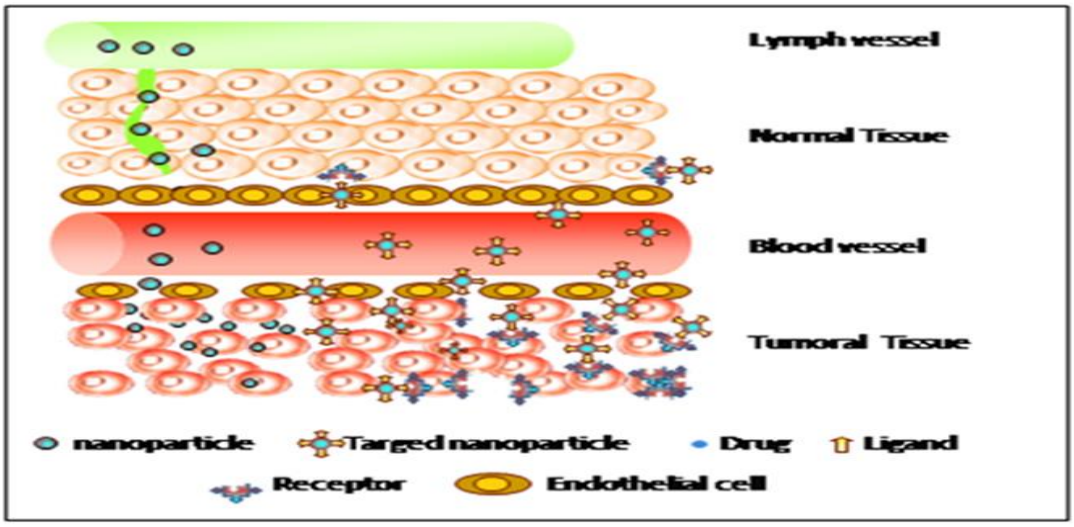


Figure 1.9. Active and passive targeting of foreign macromolecules (Blanco et al., 2012)

One of the major problems with this nanotherapeutic drug carrier is that they are rapidly eliminated from the blood stream through phagocytosis by the reticuloendothelial system (RES) (Stolnik et al., 1995, Owens and Peppas, 2006, Bawa, 2008). Avoidance of phagocytosis can be achieved through PEGlyation (surface modification, surface coating of PNP) using polyethylene glycol (PEG), i.e. formation of the so-called "stealth" PNPs,

which abrogate the rapid uptake by phagocytic cells (Greenwald et al., 2003, Jokerst et al., 2011). PEGylation can shield active ingredients from recognition and degradation by the immune system, can lower their renal filtration and prolong their circulation half-life. Therefore, a significant reduction of the overall dosage or frequency of drug administration will be achieved. By providing a hydration layer and steric barrier surrounding the polymeric core, PEG grafting can reduce non-specific binding of serum proteins to the particles, thereby reducing their clearance by cells of the mononuclear phagocytic system. PEGylation technology has the advantages of delivery natural unstable therapeutic proteins with short circulating half-lives, such as enzymes, hormones, and antibodies (Jokerst et al., 2011, Ledet and Mandal, 2012). PEG has been the most common hydrophilic blocking agent used since it is readily hydrated; it has a high degree of conformational flexibility and is biocompatible. It is approved for internal use in human by the United State Food and Drug Administration (US FDA)(Gref et al., 1994, Luo et al., 2002). The inner hydrophobic block has shown greater variability depending on the system being studied (Jee et al., 2012). Figure 1.10 shows PEGlyation with different PNs formulations(Hafner et al., 2014).

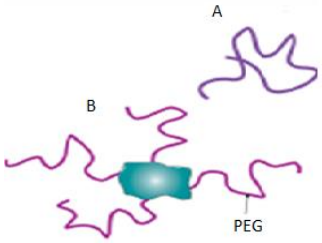
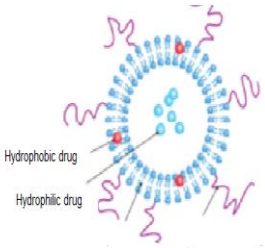
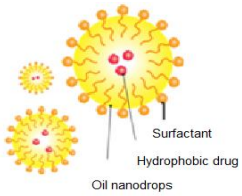
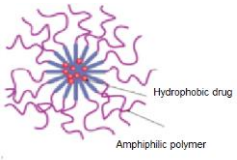
Polymeric therapeutic	Liposomes
Water soluble polymers, either as a bioactive itself (A) or as an inert functional part of a multifaceted construct for improved drug, protein or gene delivery (B). Size < 25 nm 	Vesicles composed of one or more concentric bilayers of lipid molecules (entrapping hydrophobic drugs) enclosing one or more aqueous com-partments (entrapping hydrophilic drugs). Size 20-30 nm 
Nanoemulsions	**Polymeric micelles**
Oil nanodroplets dispersed within aqueous continuous phase suitable for entrapment of hydrophobic drugs Size: 20–200 nm 	Supramolecular aggregates composed of amphiphilic block copolymers that self-assemble into aqueous media; inner core typically serves as a container for hydrophobic drugs Size: 20–80 nm 

Figure 01.10. Examples of PEGlyation of nanotherapeutics (Hafner et al., 2014).

Examples of alternative hydrophilic polymers for nanoparticle stealth functionalization rather than PEG are presented in Figure 1.11 (Hu et al., 2013) as poly (vinyl alcohol) (PVA), poly(4-acryloylmorpholine), poly (N,N-dimethylacrylamide) and poly(N-vinyl-2-pyrrolidone) (PVP) (Hu et al., 2013).

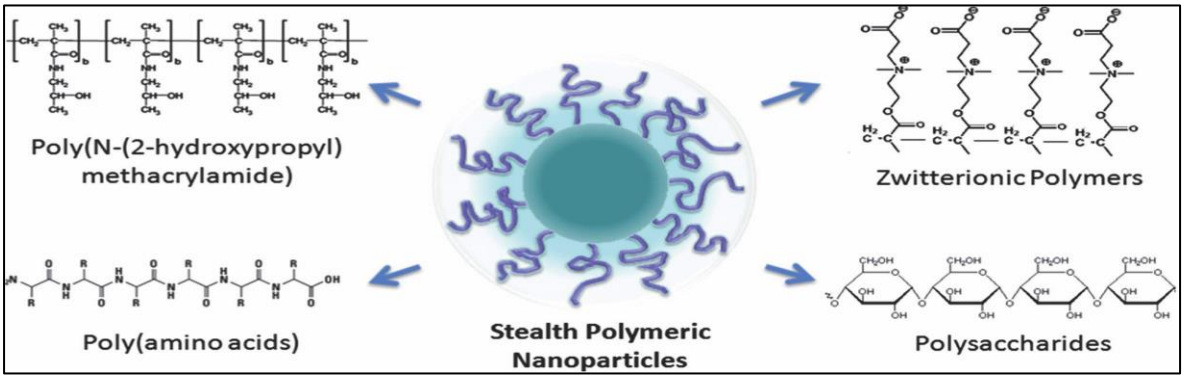


Figure 1.11. Examples of alternative hydrophilic polymers for nanoparticle stealth functionalization rather than PEG (Hu et al., 2013)

PNPs are characterized by larger surface area which improves bioavailability, unlikely to cause blood vessels blocking and improved therapeutic response. In addition, PNPs, are used as controlled and sustained release of the drug either during drug movement or at the site of action affecting its organ distribution and reduce its renal clearance leading to improving drug efficacy and reducing its adverse effects (Jawahar and Meyyanathan, 2015). The PNPs system can be used for various routes of administration, including oral, nasal, parenteral, intra‑ocular, etc(Greenwald et al., 2003, Jawahar and Meyyanathan, 2015).

1.2. Polymeric Nanoparticles

PNP scan be classified according to method of preparation into two categories; nanocapsules and nanospheres which are shown in Figure 1.12 (Jawahar and Meyyanathan, 2015). Nanocapsules are reservoir in which a liquid or semisolid drug-loaded core is enclosed in a polymeric coat. While nanospheres are matrix systems in which a drug is uniformly distributed through solid polymer matrix (Brigger et al., 2002, Vauthier and Bouchemal, 2009). The term "nanoparticles (NPs) is adopted because it is often very

difficult to ambiguously establish whether these particles are of the matrix or a membrane type (Branch, 1988, Quintanar-Guerrero et al., 1998a, Fattal and Vauthier, 2002, Jeong et al., 2002, Ochekpe et al., 2009, Vauthier and Bouchemal, 2009, Kumari et al., 2010, Parveen et al., 2012).

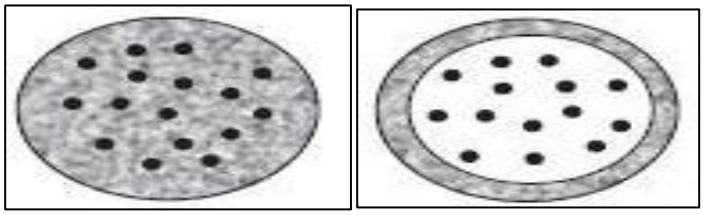


Figure 1.12. Spherical polymeric nanoparticles either nanosphere (left) or nanocapsule (right) (Jawahar and Meyyanathan, 2015)

Furthermore PNPs for pharmaceutical applications drug delivery systems as shown in Figure 1.13, can be classified based on their special orientation in the space; into zero, one, two and three dimensions (Hett, 2004, Konwar and Ahmed, 2013). One dimensional system, such as thin film or manufactured surface, has been used for decades in electronic and chemical engineering. One dimensional PNP include carbon nanotubes (CNTs); it is hexagonal network of carbon atoms, 1.0 nm in diameter and 100 nm in length, as a layer of graphite rolled up into cylinder. Three dimensional PNPs include Fullerenes (Carbon 60), which consist of spherical cages containing from 28 to more than 100 carbon atoms, contain C_{60}. This is like a hallow ball, resemble a soccer ball(Konwar and Ahmed, 2013).

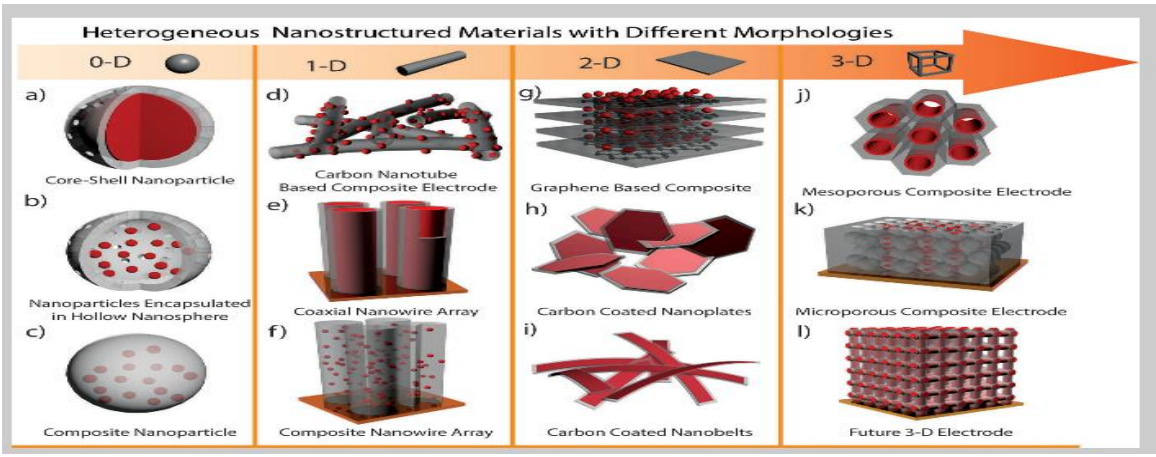


Figure 1.13. Heterogonous PNPs based on their structural complexity; zero-dimensional (0-D), one-dimensional (1-D), two-dimensional (2-D), three-dimensional (3-D) and the even more complex chiral 3-D nano structural networks (Konwar and Ahmed, 2013).

The benefits of using PNPs, as a drug delivery system, include: increase of the solubility, protection against degradation, prolonged release, improved bioavailability, targeted delivery and decrease the toxic side effects of drugs. They also offer appropriate form for all routes of administration and allow rapid-formulation development (Mohanraj and Chen, 2007, Parveen et al., 2012). However, PNPs have some use constraints especially in liquid form due to particle-particle aggregation because of their small size and large surface area. In addition, PNPs have lower drug loading and show burst release. These real-world problems have to be solved before PNPs are being available in the market for clinical utilization(Mohanraj and Chen, 2007, Parveen et al., 2012).

The ideal polymer for PNPs preparation has to be biodegradable, completely eliminated from the body in a reasonable time, long stability in circulation, and apparent lack of immunogenicity(Vilar et al., 2012). The polymer must also be compatible with different drugs, leading to high drug loading, possesses appropriate properties for easier fabrication and its degradation products have to be nontoxic(Mohanraj and Chen, 2007, Parveen et al., 2012). To date only a few of the polymers available are approved for drug delivery and *in vivo*use (Vauthier and Bouchemal, 2009).

The final PNPs delivery systems should be simple and inexpensive, reproducible and stable. No toxic organic solvents should be involved in their formulations. They are appropriate to various groups of drugs; small molecular weight drugs as well as proteins and polypeptides. They are easy to handle and have the ability to be lyophilized and stable after reconstitution with the suitable vehicle(Nagavarma et al., 2012).

PNPs are successfully employed to a wide range of research fields as anticancer, anti-inflammatory, immunosuppressant, hormones, antifungals, and vitamins, etc. PNPs can improve the efficacy of different drugs; cover nasty taste, prolong their release, improve bioavailability and protect these drugs from degradations(Nagavarma et al., 2012).

1.2.1. Polymer Types:

Despite the structure and function diversity of polymers, they are classified in a number of ways depending upon various criteria as shown in Figure1.14 (Vollmert and Immergut, 1973, Stevens, 1990, Fried, 2014). On the basis of source or origin, the polymers

are classified into three types: natural polymers, semi-synthetic polymers and synthetic polymers. Natural polymers (biopolymers) are found in nature, mostly obtained from plants and animal sources and they include polysaccharides (starch and cellulose), proteins (α-amino acids, wool, natural silk, leather, etc.), nucleic acids (RNA and DNA) and natural rubber(2-methyl buta-I, 3-diene (isoprene). These natural polymers have been widely utilized in drug delivery systems due to their intrinsic biodegradability and biocompatibility. Some disadvantages of their use are poor batch to batch reproducibility and their potential antigenicity (Mohanty et al., 2015).

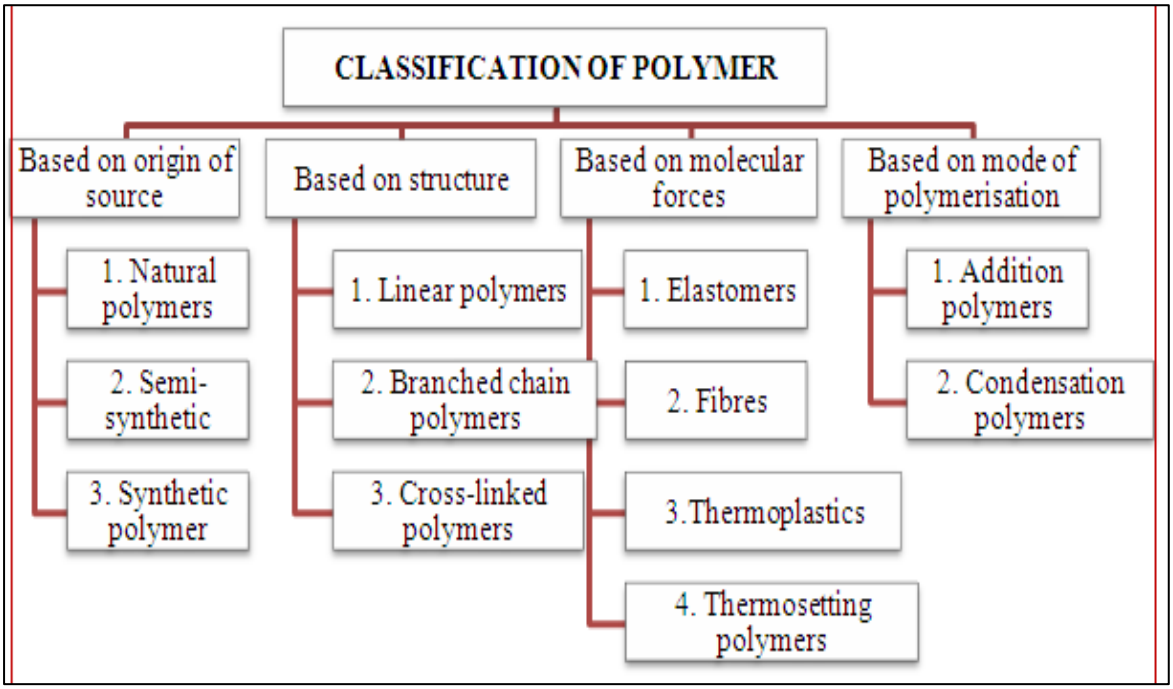


Figure 1.14. Classification of polymer (Fried, 2014)

Semi-synthetic polymers are mostly derived from naturally occurring polymers with further chemical alterations as cellulose derivative. The most important polymers in this class are known as cellulose ethers that can be further classified as nonionic (including methyl cellulose, hydroxyethyl cellulose, etc.) and anionic (primarily sodium carboxymethyl cellulose). Chitin and starch are derivatized to form water-soluble polymers of this class(Mikkelsen, 1994). Chitosan polymers are semisynthetic polymers derived from deacetylation of their parent polymer chitin. They have unique structures, multidimensional

properties, highly sophisticated functionality and a wide range of applications in biomedical and other industrial areas(Dash et al., 2011).

Synthetic polymers or man-made polymers as polyethylene, polystyrene, polytetrafluoroethylene, synthetic rubber, nylon, polyvinyl chloride, bakelite, teflon, orion, etc. The synthetic polymers can be further classified as these made up of monomers and comonomer units. The main limitation of these carriers is the presence of toxic residues such as unreacted free monomer, stabilizers, crosslinking agents, residues from initiators surfactants and processing aids, whose elimination requires time-consuming and inefficient procedures (Czech and Wesolowska, 2007). In addition, the anionic nature of the monomers may result in a cross-reaction between drug molecules and the acrylic monomer. These drawbacks led to the development of methods involving the use of well-characterized preformed polymers(Fried, 2014).

More polymers classification can be based on particular considerations (Figure 1.14, (Fried, 2014) as structure, molecular forces and mode of polymerization. Additional reviews of polymers classification are been published by Vollmert et al., Stevens, and Fried (Vollmert and Immergut, 1973, Stevens, 1990, Fried, 2014).

Polymers classification based on their structures include; linear, branched cross-linked and network polymers. Figure 1.15(Anadão, 2012)shows schemes of these types of polymers. Linear polymer is consisting of a single continuous chain of repeated units. Branched chain is a polymer with side chains of repeated units connecting into the main chain of repeated units. Cross-linked polymer includes interconnection between chains, while network polymer is a cross linked polymer that includes numerous interconnections between chains.

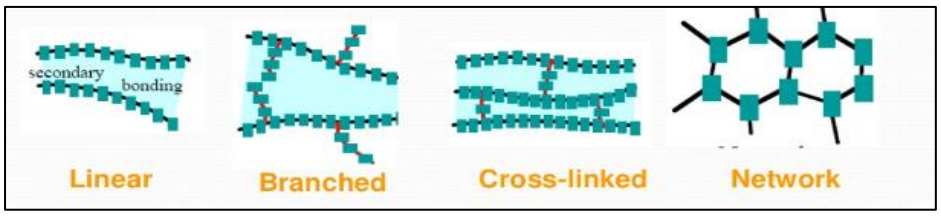


Figure 1.15. Schematic classification of polymer based on structure(Anadão, 2012)

Additionally, polymer can be classified based on molecular forces include; elastomer (rubber), fibers, thermoplastics and thermosetting polymers(Anadão, 2012).

In drug delivery systems, biodegradation plays a major role, so, polymers are further classified into two subgroups as biodegradable and non-biodegradable. Biodegradable polymers are defined as polymers, which degrade *in vitro* and *in vivo* either into products that are normal metabolites of the body or into products that can be eliminated from the body with or without further metabolic transformation. The degradation products should be nontoxic, and the rate of degradation and mechanical properties of the material should match the proposed claim. Non-biodegradable hydrophobic polymers initially used for drug delivery applications are cellulose derivatives, acrylic polymers, silicones and others (Polyvinyl pyrrolidone, ethyl vinyl acetate, poloxamers, poloxamines) (Pillai and Panchagnula, 2001). Degradable polymers do not need surgical elimination and hence are favored to non-biodegradable polymers in several uses (Bala et al., 2004). Thus, both natural and synthetic biodegradable polymers have been extensively investigated for drug delivery applications.

Natural biodegradable polymers, biopolymers, are biocompatible but some may suffer from antigenicity, unstable material supply and batch-to-batch variation(Pillai and Panchagnula, 2001). The natural polymers which are commonly used in drug delivery systems include polysaccharides (starch, cellulose, chitin, chitosan, alginic acid), cellulose esters, as cellulose acetate; modified polysaccharides, and proteins (gelatin, elastin, albumin, fibrin and soy protein) (Roy et al., 2009).

While, synthetic biodegradable polymers are easy to modify and control their degradation with high reproducibility. Semi-synthetic biodegradable polymers; the fermentation of sugars produces different monomers. Polylactic acid (PLA) is synthesized from lactic acid produced via starch fermentation from lactic bacteria. Carbohydrate polymers such as xanthan, curdlan, pullulan and hyaluronic acid, produced by fermenting a sugar feedstock with bacteria or fungi(Vilar et al., 2012).

The most widely used biodegradable polymers are synthetic polymer. They include; polyesters, polyamides, polyurethanes and polyureas, poly (amide-enamines), polyanhydrides. The polyesters polymer include- poly (glycolic acid) (PGA), poly(lactic acid)(PLA), poly (alkenedicarboxylate), poly (butylene succinate), poly(ethylene succinate), polycaprolactone (PCL), poly(butylene succinate) (PBS) and its copolymers.

Polyesters are the utmost common and popular biodegradable polymers since their use as biodegradable sutures. PGA, PLA and their correlated copolymer poly (D, L-lactide – co-glycolide) (PLGA). Their biodegradation is commonly achieved through hydrolysis of the ester bond resulting in nontoxic inactive degradation products. Polyesters are the only group approved by Food and Drug administration (FDA) for clinical use as carriers for different drugs to prolong their release and improve their *in vivo* performance (Parveen and Sahoo, 2008). They are applied to transdermal delivery systems (Kandavilli et al., 2002), oral dosage forms, osmotic pumps(Wong et al., 1986), swellable hydrophilic polymer matrices(Korpman, 1984) and parenteral dosage forms(Packhaeuser et al., 2004).

From the solubility perspective, pharmaceutical polymers can be categorized as water-soluble and water-insoluble (oil soluble or organic soluble). Water-soluble synthetic polymers are poly (acrylic acid), poly (ethylene oxide), poly (ethylene glycol, PEG) (Figure 1.16), poly (vinyl pyrrolidone, PVP), poly (vinyl alcohol, PVA), hydroxypropyl methyl cellulose (HPMC). They have extensive applications in pharmaceutical industries to improve drug bioavailability (Sheen et al., 1995, Sugimoto et al., 1998, Zhang et al., 2014b). PEG is the most common used polymer for the drug delivery applications and is widely used as drug carrier for various types of drugs. It is known that low molecular weight PEGs are easily excreted in humans.

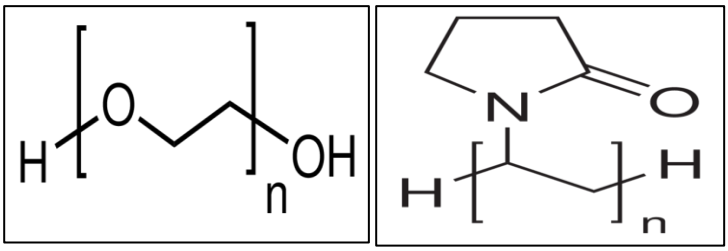


Figure 1.16. Molecular structures of PEG 4000 (left) and PVP (right) (Chen et al., 2012)

The most used polyesters are the water insoluble biodegradable polymers including; PLA, PGA and PLGA. Table 1.2 shows examples of different drug delivery systems in the market for drugs utilizing these polymers for various diseases. Anticancer drugs such as cisplatin, doxorubicin and 5-Fluorouracil have been encapsulated using a biodegradable polymer such as PLGA. Drugs release profiles and degradation behaviors of these NPs have favorable drug release profiles with a short initial burst release, less toxic effect and long release time (Ike et al., 1991, Huo et al., 2005, Kumari et al., 2010, Wohlfart et al., 2011). Additionally, numerous studies have focused on polymer carriers (PLGA) that encapsulate peptide hormones and various antibiotics (Sinha and Khosla, 1998, Jain, 2000, Gavini et al., 2004, Kumari et al., 2010). The chemical structures of PLA, PGA and PLGA are shown in Figure 1.17(Huh et al., 2003).

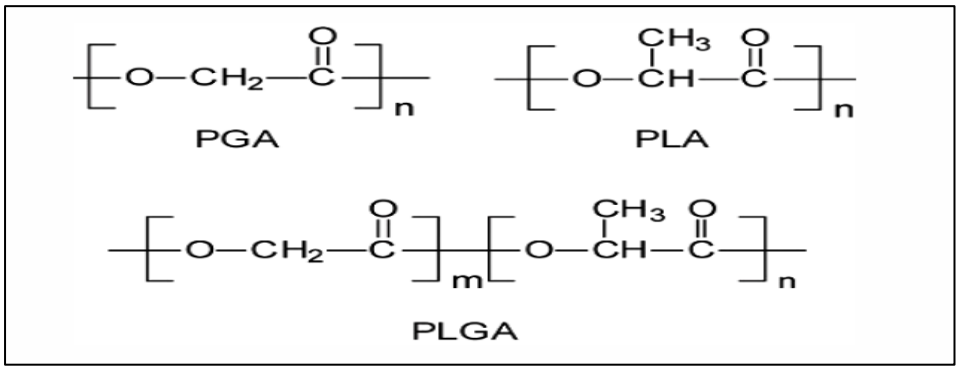


Figure 1.17. Chemical structure structures of PLA, PGA and PLGA (Huh et al., 2003)

Table 1.1. Commonly used polymers for drug delivery applications

Polymer Name	Polymer Abbreviation	Drug	Application or Use
Poly(lactide)	PLA	Haloperidol	antipsychotic
		Dexamethasone	Rheumatoid arthritis
Poly(lactide-co-glycolide)	PLGA	Taxol	Anticancer
		Paclitaxel	Anticancer
Poly(ε-caprolactone)	PCL	Tamoxifen	Anticancer
		Clonazepam	antipsychotic
Gelatin		Paclitaxel	Anticancer
Chitosan		Cyclosporin A	immunosuppressant

*(Kumari et al., 2010)

Various studies have been focused on PLGA as a drug carrier due to its long history in medicine as the material for biodegradable surgical sutures (Gilding and Reed, 1979, Oh, 2011). It is emerged as the most promising material for drug delivery nanocarriers fabrication.

PLGA copolymers undergoes degradation in aqueous environment (hydrolytic or biodegradation)*in vitro* and *in vivo* through cleavage of backbone ester linkage. The degradation behavior of polymer is also dependent on hydrophobicity of the polymer; the more hydrophobic the polymer is the more slowly its degradation. The hydrophobicity of the polymer is affected by the ratio of crystalline to amorphous sections, which in turn is determined by copolymer configuration and monomer stereochemistry (Mittal et al., 2007). Lactic acid is more hydrophobic than glycolic acid. It is known that PLGA biodegrades to form lactic acid and glycolic acid as shown in Figure 1.18(Kumari et al., 2010). Since these two monomers are endogenous and definitely metabolized by the body via the Krebs cycle, a negligible systemic toxicity is linked with the usage of PLGA in drug delivery or biomaterials applications (Kumari et al., 2010). Lactic acid enters tricyclic acid cycle (TCA) and subsequently metabolized to CO_2 and H_2O and eliminated from the body. Glycolic acid is excreted unchanged in the urine or it enters TCA cycle and is eliminated as CO_2 and H_2O. US FDA and European Medicine Agency (EMA) have approved the use of PLGA in various drug delivery systems in humans (Administration, 2007).

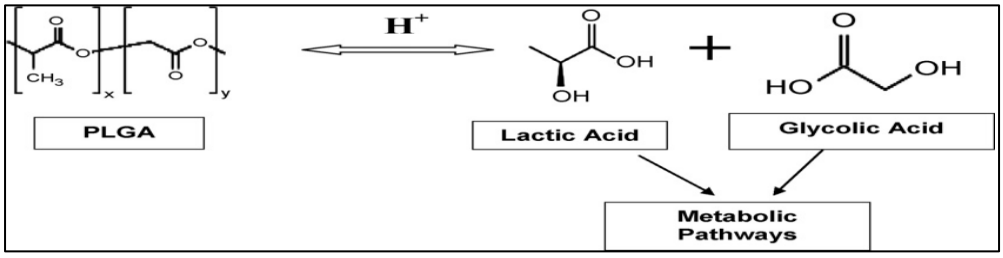


Figure 1.18. Hydrolysis of PLGA NPs(Kumari et al., 2010)

The size of NPs make them easily target for capture by macrophages of liver and spleen "reticuloendothelial system (RES)" leading to shorter circulation half-life(Bala et al., 2004) and constrain their reaching of other organs in the body. Hydrophilic coating of the surface of NPs with PEG could modify their biodistribution and uptake with providing protection against macrophage and therefore, prolong their circulation time.

Gref et al., were the first to describe the effect of PEG on the pharmacokinetics of PLGA microparticles externally coated with PEG. The authors demonstrated that a radical increase in half-life of micelles has occurred when these were covered by PEG polymer. Only 30% of these micelles were captured by liver after 2 h of injection (Gref et al., 1994). Since that, PLGA–PEG were extensively investigated for various applications like implantable materials (Yu and Ding, 2008); pharmaceutical products(Kamaly et al., 2012), drug delivery systems (Cheng et al., 2007), and the market are expected to continue to grow further for more application of PLGA – PEG (Kamaly et al., 2012).

PLGA-PEG copolymer shows quite different properties when compared to each constituting polymers so it is a new and unique biomaterial. It possess both the biodegradability and biocompatibility of PLGA polymer and the stealth behavior of PEG but more than this PLGA-PEG can form micelles better than the single PLGA or other type of PLGA–PEG copolymers because of its well distinct lipophilic portion (PLGA) and its hydrophilic one (PEG) that make the PLGA-PEG an amphiphilic polymer. These two parts during micelles formation self-assemble generating a system in which the hydrophobic PLGA remains inside the micelles and the hydrophilic PEG goes outside creating a stabilizing shell. In this way, it is possible to easy entrap of lipophilic molecules like drugs or smaller nanostructures in the inner core constituting of PLGA chains. Furthermore, it is also possible a formation of residual functional groups in the outer shell by previous functionalization of the PEG chains that can be used for conjugation of active molecules (proteins, peptides, monoclonal antibodies) for targeting desired tissues. Various functional groups can be useful for this and PEG chains of various lengths already functionalized with a wide variety of reactive groups such as amino, carboxylic acid, methoxy, maleimido, etc., are commercially available (Locatelli and Franchini, 2012).

1.2.2. Amphiphilic Block Copolymers

Amphiphilic block co-polymers (ABCs) have been in use as pharmaceutical excipients in different forms for a long time. Their application is experiencing a rapid growth in modern pharmaceutical sciences (Adams et al., 2003, Letchford and Burt, 2007, Zhang et al., 2014a).

ABCs consisting of two parts a hydrophobic polyester part and a hydrophilic PEG part (Figure 1.19 (Zhang et al., 2014a)). They are considered as synthetic biodegradable and biocompatible with several block assemblies and configurations. These ABCs are biocompatible, FDA-approved for clinical use, and biodegradable by enzyme and hydrolysis under physiological conditions (Zhang et al., 2014a). Nowadays, ABCs are currently available commercially as Resomer®manufactured by Boehringer Ingelheim.

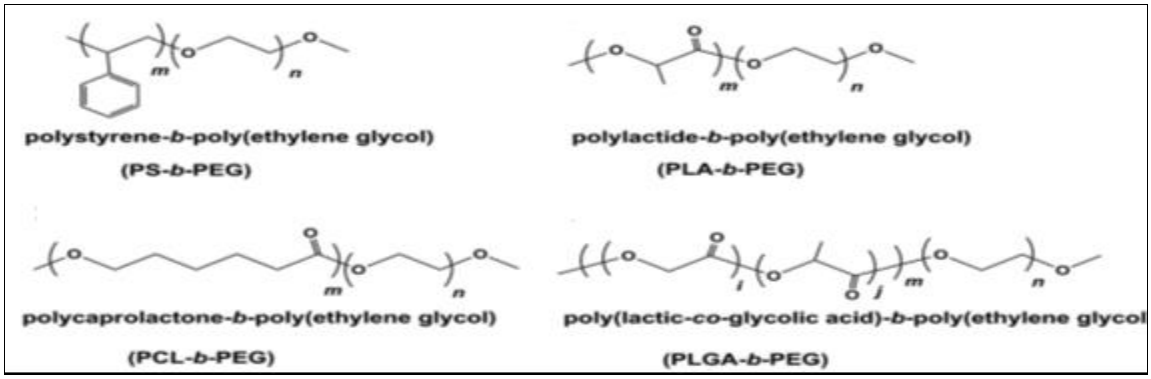


Figure 1.19. The chemical structure of some amphiphilic block copolymer(Zhang et al., 2014a)

ABC polymer has a unique self-assembly feature (Figure1.20) and can form various kinds of NPs such as polymer micelles, polymersomes and hydrogels (Zhang et al., 2014a). ABCs assemble into nano-scaled core–shell structures, micelles, which are of considerable interest for delivering drugs with poor water solubility (Shao et al., 2010a).In aqueous media, ABCs solubility is very different. Their hydrophilic and hydrophobic parts, spontaneously form polymeric micelles in which a hydrophobic inner core is surrounded by a distinct core-shell structure(Zhang et al., 2014a).

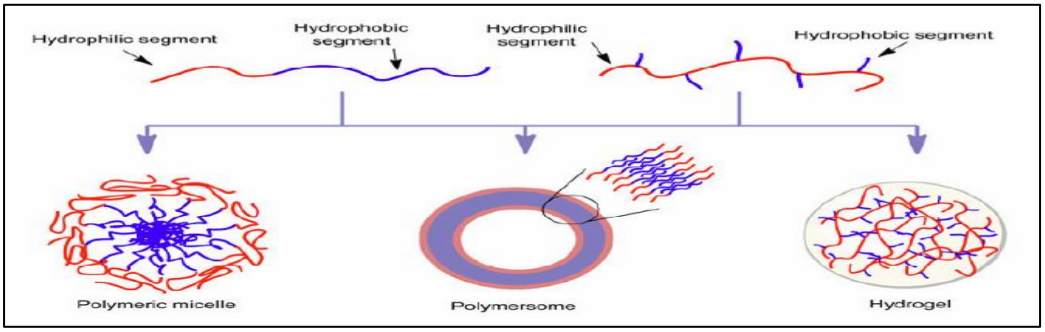


Figure 1.20. Typical structures of amphiphilic polymers and their self-assemblies: polymeric micelle, polymersome, and hydrogel (Zhang et al., 2014a)

The rapid development of ABC applications in the pharmaceutical sciences is mainly due to the chemical flexibility of their assembly, which provides an opportunity for the design of multipurpose drug carriers. Diverse types of copolymers based on PLGA and PEG were created, such as di-, tri, or multi-block copolymers. Also linear, branched, and mixed copolymers, with or without functional ending groups, were investigated, but most of all the di-block copolymer (PLGA-PEG) has attracted particular interest due to special properties, such as amphiphilicity and easier synthesis, in comparison to the others (Huh et al., 2003).

Block copolymers have been extensively developed to date and can be classified according to their block structure as AB diblock, ABA, or BAB triblock, branched block, and star-shaped block copolymers, in which A is a hydrophobic block made up of biodegradable polyesters and B is a hydrophilic PEG block, as shown in Figure1.21 (Loh and Li, 2007).

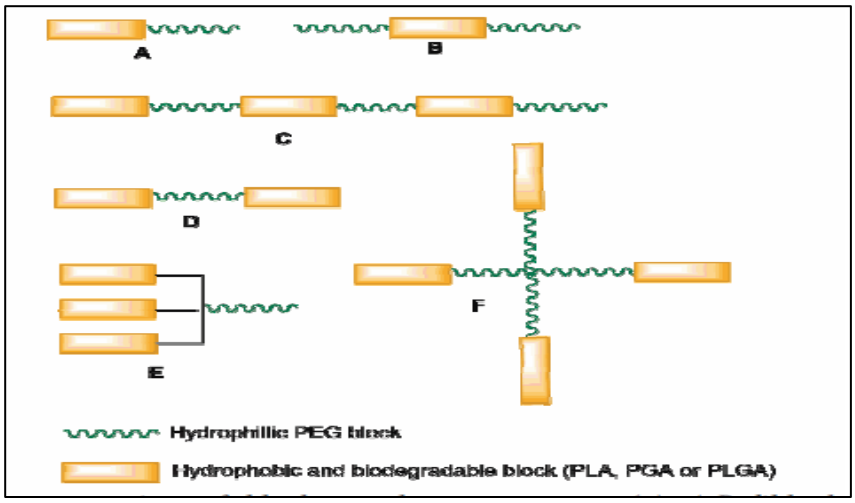


Figure 1.21. Schematic presentation of block copolymer structure; (A)-A-B diblock, (B) A-B-A, (C) B-A-B, (D) alternating multiblock, (E) muti-armed structure, and (F) star-shaped block(Loh and Li, 2007)

ABCs have been in use as pharmaceutical excipients in different forms for a long time and their application is experiencing rapid growth in pharmaceutics and in nanomedicine.

Several previous studies have shown that loading AmB to different copolymer were administered parenterally led to decreased hemolysis and/or nephrotoxicity while retaining the antifungal activity of AmB. Development of AmB loaded with copolymers such as poly(ethylene oxide)-block-poly(N-hexyl stearate l-aspartame), poly(ethylene oxide)-block-poly(-benzyl-l-aspartate), PEG-p (caprolactone-co- trimethylenecarbonate), poly(2-ethyl-2-oxazoline)-block-poly(aspartic acid), (PEG)3-PLA, poly(ethylene oxide)-poly(_-caprolactone) and poly(isoprene-b-ethylene oxide) (Vandermeulen et al., 2006, Wang et al., 2009, Jain and Kumar, 2010, Falamarzian and Lavasanifar, 2010b, Pippa et al., 2014).

Additionally for the oral delivery of drugs, several published papers showed there were increased in bioavailability when loading different drugs with either di, or tri-block copolymer, such as insulin loaded to PLGA-PEG di- and triblock copolymer (Wang et al., 2009) curcumin (Pippa et al., 2014) and oral vaccines (Garinot et al., 2007).

1.3. Polymeric Nanoparticles Preparation Methods

For the preparation of PNPs drug delivery systems, there are different methods employed: solvent evaporation, nanoprecipitation, single emulsion-solvent evaporation, double emulsification, salting out, emulsification solvent-diffusion method and spray drying technology (Asher and Schwartzman, 1977, Nagavarma et al., 2012, Jawahar and Meyyanathan, 2015). Figure 1.22shows the common technique of the different methods for the preparation of these NPs(Bennet and Kim, 2014). Nevertheless, other methods have been used for NPs formulations. There are reviews discussing in details these methodologies concerning polymers' selection and the mechanistic fundamentals of each methods (Singh and Muthu, 2007, Mora-Huertas et al., 2010).

The selection of the method of preparation is affected by the physicochemical properties of the drugs, mainly its solubility and the therapeutic use of PNPs also the route of administration and drug release profile. Method of preparation also can be affected by drug stability, stirring speed, solvent used, drug encapsulation efficiency, method reproducibility, washing step and time consumption(S en et al., 1998).

The nanoprecipitation method (solvent displacement) according to Fessi et al. (Fessi et al., 1988), consisted of two phases; organic and aqueous phases. Evaporation under

reduced pressure is essential to remove the organic solvents and then NPs are recovered by a suitable purification process such as centrifugation (Fessi et al., 1988, Barichello et al., 1999, Singh and Muthu, 2007, Nagavarma et al., 2012, Konwar and Ahmed, 2013).

Solvent evaporation method in which, the polymer is dissolved in organic solvent with or without drug and the resulting mixture is emulsified into a surfactant-containing aqueous solution by sonication. Evaporation of the organic solvent and recover the NPs by centrifugation or dialysis (Mora-Huertas et al., 2010, Nagavarma et al., 2012, Konwar and Ahmed, 2013).

Single emulsion-solvent evaporation method is primarily used for entrapping lipophilic/hydrophilic drugs. In this method, the drug and polymer are first dissolved in a volatile organic solvent such as chloroform or dichloromethane. The drug-polymer solution is emulsified into an aqueous phase containing an emulsifier such as poly (vinyl alcohol) (PVA), gelatin, polysorbate 80, or polaxamer-188 to form an o/w emulsion. Subsequently, the formulation is subjected to organic solvent removal/ extraction to harden the nanoemulsion droplets into solid NPs (Bennet and Kim, 2014). Reduction of the NPs size is achieved by either sonication or high-speed homogenization. Evaporation of organic solvent at higher temperature and under pressure with continuous stirring for several hours. The time needed for solvent evaporation has significant effects on the porosity of the NPs, and then affects the drug release (Arshady, 1991). Poor drug loading and burst release effect of hydrophilic drugs are the most disadvantages of this method (Konwar and Ahmed, 2013, Mora-Huertas et al., 2010, Nagavarma et al., 2012).

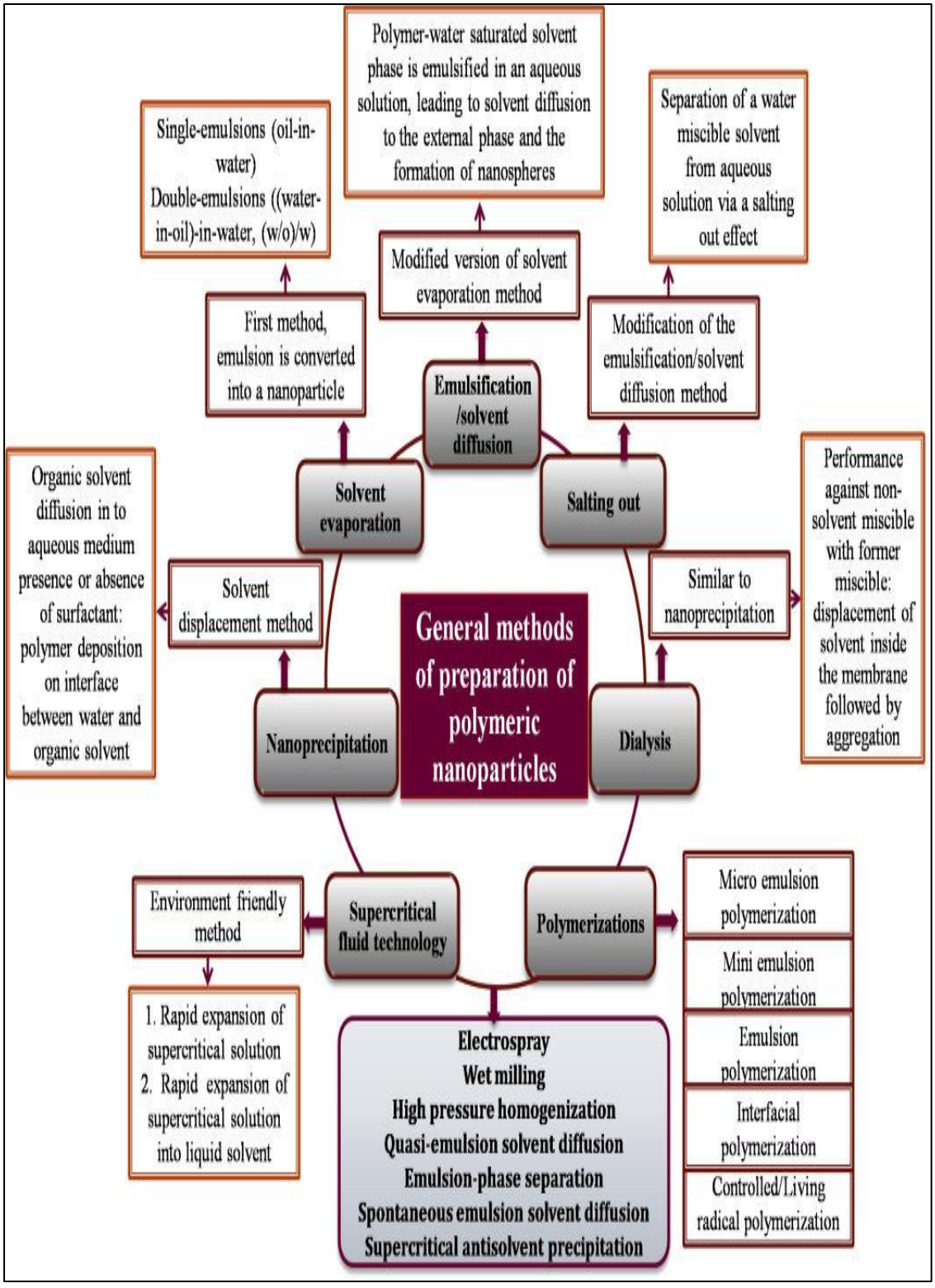


Figure 1.22. General methods of preparation of polymeric NPs (Bennet and Kim, 2014)

Double emulsion (water/oil/water "w/o/w") solvent evaporation is the technique that commonly involves the formation of a primary water/oil (w/o) emulsion by sonication organic phase of the polymer, with/without the drug, and aqueous phase. A surfactant containing aqueous solution is then added to the primary emulsion with sonication to obtain the double emulsion (w/o/w). Evaporation for the organic solvent either at room temperature or under reduced pressure. Recovery of the NPs either by centrifugation or dialysis (Mora-Huertas et al., 2010, Nagavarma et al., 2012, Konwar and Ahmed, 2013).

Salting out is composed of the addition of either highly electrolyte or a non–electrolyte containing stabilizer to organic phase composed of polymer and the drug. After the preparation of the initial w/o emulsion, then phase inversion to o/w emulsion is achieved by adding sufficient amount of water. This induces complete diffusion of organic solvent from the organic phase into the continuous external aqueous phase, followed by solvent removal and recovery of the NPs (Allémann et al., 1993, Vauthier and Bouchemal, 2009).

Emulsification solvent-diffusion method is technique that was developed by Dueler and coworker(Quintanar-Guerrero et al., 1996), which involved emulsification of drug-polymer solution in partially water-miscible solvent (such as ethyl acetate, benzyl alcohol, propylene carbonate) pre-saturated with water, in an aqueous solution containing stabilizer under vigorous stirring to form o/w emulsion. The process is followed by reduction in droplet-size using a high-speed homogenizer/sonicator (Quintanar-Guerrero et al., 1996). Subsequently, water is added to the o/w emulsion system under constant stirring causing outward diffusion of the organic solvent from the internal phase, which leads to the nanoprecipitation of the polymer. Finally, the solvent can be eliminated by evaporation or vacuum distillation(Mora-Huertas et al., 2010, Nagavarma et al., 2012, Konwar and Ahmed, 2013). One of the main disadvantages of this method is low entrapment efficiency for hydrophilic drugs, addition of specific lipids to the aqueous phase could improve this limitation (Bala et al., 2004).

Spray drying offers a relatively rapid and convenient production of NPs that is easy to scale-up, involves mild processing conditions and has relatively less dependence on the solubility characteristics of the drug and the polymer. In spray drying technique, a solution

of a drug in an organic solvent containing the polymer is sprayed from the sonicating nozzle of a spray dryer and subsequently dried to yield NPs (Mora-Huertas et al., 2010, Nagavarma et al., 2012).

Dialysis, is very simple and effective method for preparing of PNs with small, narrow size distribution(Nagavarma et al., 2012). Organic solvent contain polymer is subjected to dialysis tube. Dialysis is performed against a non-solvent miscible with the former miscible. The displacement of the solvent inside the membrane is followed by the progressive aggregation of polymer due to a loss of solubility and the formation of homogeneous suspensions of NPs.

One of the fast and reproducible methods for NPs preparation is the emulsion polymerization. It is broadly classified to an organic or aqueous continuous microemulsion. Dispersion of monomer into an emulsion or inverse microemulsion, or into nonsolvent led to formation of continuous organic phase (Nagavarma et al., 2012). Disadvantages of this method it is the existence of toxic organic solvents, surfactants, monomers and initiator, during preparation process which, then should be removed from the designed NPs, additionally usage of non-biodegradable polymer had limited the preparation of NPs by this method.

Purification is the last step required for all previous mentioned NPs preparation techniques. One of the following method such as ultracentrifugation, ultrafiltration, gas chromatography, dialysis, spray drying, lyophilization or a combination of these methods could be used at the purification step.

Emulsion diffusion evaporation or nanoprecipitation is the most suitable method for hydrophobic drugs' preparation while double emulsion method is appropriate for hydrophilic drugs, such as proteins and peptides(Quintanar-Guerrero et al., 1996, Quintanar-Guerrero et al., 1998b, Mora-Huertas et al., 2010, Nagavarma et al., 2012).

1.3.1. Emulsification-Diffusion Method

The emulsification-diffusion method (ESD) has been used successfully to prepare biodegradable NPs in an effective and reproducible way especially for hydrophobic drugs. However, double emulsion method is suited for hydrophilic drugs, such as proteins and peptides (Quintanar-Guerrero et al., 1998a, Quintanar-Guerrero et al., 1998b, Mora-Huertas et al., 2010, Nagavarma et al., 2012). The polymer, the drug and an organic solvent which are incompletely miscible with water and which should be water-saturated. This organic medium acts as solvent for the different components of the organic phase. The aqueous phase encompasses the aqueous dispersion of a stabilizing agent that is prepared using solvent-saturated water while the dilution phase is usually water as seen in Table 1.3 and Figure 1.23(Mora-Huertas et al., 2010).

Table 1.3. Suggested composition for preparation of nanocapsules by emulsion–diffusion method*

Material	Suggested composition
Active substance	10–50mg
Polymer	1.0–2.0% of inner phase solvent
Oil	2.5–5.0% of inner phase solvent
Inner phase solvent	10 ml
Stabilizer agent	2.0–5.0% of external phase solvent
External phase solvent	40 ml
Dilution phase	200 ml

*(Mora-Huertas et al., 2010)

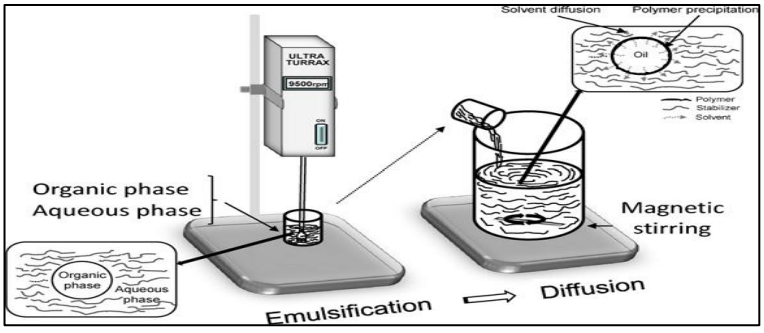


Figure 1.23. Set-up used for preparation of nanocapsules by the emulsion–diffusion method (Mora-Huertas et al., 2010)

This method is characterized by scale up simplicity, reproducibility, high encapsulation efficiencies (>70%), no need for homogenization, and narrow particle size distribution (Nagavarma et al., 2012). Time consuming is one of the disadvantages of this method since elimination of large amount of water from the suspension takes long time and some leakage of water-soluble drug into the saturated-aqueous external phase during emulsification may take place(Reis et al., 2006, Mohanraj and Chen, 2007). It is efficient method for encapsulating lipophilic drugs (Quintanar-Guerrero et al., 1998a). Various drug-loaded NPs were made by the ESD method, including mesotetra (hydroxyphenyl) porphyrin-loaded PLGA (p-THPP) NPs(Vargas et al., 2004), doxorubicin-loaded PLGA NPs(Yoo et al., 1999), plasmid DNA-loaded PLA-NPs(Perez et al., 2001), coumarin-loaded PLA NPs(Lu et al., 2005), indocyanine and with other drugs (Nagavarma et al., 2012).

Depending on their composition, physicochemical properties and biopharmaceutical behavior, NPs may be designed and applied to several routes of administration, such as oral, parenteral, topical, nasal, among others (Heurtault et al., 2003). Although each route of administration has its own advantages, the oral route is widely known as the most convenient one for the treatment of chronic conditions. However, the delivery of 50% of drugs by this route is limited due to the great lipophilicity of the drugs themselves and approximately 40% of new drug candidates present low solubility in water leading to poor bioavailability and high intra- and inter-subject variability and deficiency of dose proportionality (Gursoy and Benita, 2004).

Drug release from the NPs is a factor of the cross‑linking, morphology, size, density of the particulate system and the physiochemical properties of the drug, as well as existence of additives(Jawahar and Meyyanathan, 2015). *In vitro* drug release is also determined by pH, polarity and occurrence of enzymes in the dissolution medium. Figure1.24 shows the three common physicochemical mechanisms at which drug is released from the polymer (Jawahar and Meyyanathan, 2015). The first mechanism involves hydration then swelling of the polymer, followed by release via diffusion of drug from the entrapped inner core. The second mechanism is through enzymatic reaction causing degradation of the polymer and the release of drug at the intended site. The third

mechanism is separation of the drug from the polymer and its release from swelled NPs. In the most cases, drug release tails more than one kind of mechanism (Jawahar and Meyyanathan, 2015). When the drug loaded to NPs subjected to dissolution medium, the drug instantaneously dissolves and release from the surface (a burst effect). Away from the surface, the drug release is through diffusion, by swelling of the matrix first, followed by transformation of the glassy polymer into a rubbery matrix and finally by diffusion of the drug from the rubbery matrix.

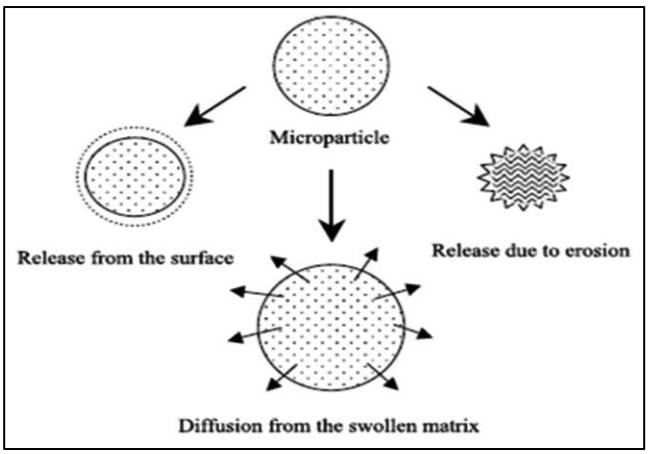


Figure 1.24. Drug release mechanisms in nanoparticles (Jawahar and Meyyanathan, 2015)

1.4. Absorption Enhancers

The selection of proper absorption enhancers, for a successful polymeric NPs formulation with high drug loading, is another challenge in NPs formulation improvement approach. For oral dosage forms delivery, absorption enhancers can be a viable formulation option in improving drug permeability, solubility and hence bioavailability(Khadka et al., 2014).

Recently, extensive researches have been published regarding the improvement of poorly absorbed drugs administration via oral, rectal or nasal route with the utilization of absorption enhancers. Different classes of compounds have been investigated as absorption enhancers such as bile salts, cyclodextrins, fatty acids, lipids, polyvinyl pyrolidone (PVP), pluronic and vitamin-E (TPGS)(Aungst, 2012).These enhancers can be added to the formulations either during the preparation or just before administrations (Aungst, 2012).

The mechanisms of absorption enhancement could involve preventing degradation/ metabolism as for peptides and protein drug molecules. This could be achieved by encapsulation of the drug to protect it, by including a protease-inhibiting excipient in the formulation, or by controlling the pH of the environment where the drug is released(Aungst, 2012). Addition of an excipient that modulates membrane permeability will increase permeability of compounds with poor membrane permeability. Any alteration in the tight junction (distension and constriction) available in the gastrointestinal and nasal epithelial membranes will alter the movement of water and low-molecular weight molecules. Another alternative mechanism of permeation enhancement involves stimulating the transcellular permeation of drugs. This requires disturbing the structure of cellular membrane e.g. surfactants. Another mechanism that has been suggested for improving absorption is the formation of a membrane permeable complex, with one type of complex being an ion pair. Solvent drag when there is coupling of solute and solvent flow through transmembrane with hydrostatic pressure or osmotic pressure gradients. The studies indicate that either the solute or solvent drag can influence the "passive transport" of solutes across the gastrointestinal tract(Aungst, 2012).

Bile salts are considered as emulsifiers of lipid compound. These biological surfactants involved in the solubilization and delivery of lipophilic drugs from the GI(Faustino et al., 2015).They accelerate lipolysis and transport of lipid products through the undisturbed water layer of the intestinal mucosa by micellar solubilization (Trotta et al., 2003, Faustino et al., 2015).

Shaikh coauthors (Shaikh et al., 2012) have investigated Cyclodextrins for their unique structures, of having hydrophilic external surface and hydrophobic cavity. They pointed out that cyclodextrins are capable of forming inclusion complexes with various drugs. Therefore, the oral bioavailability of poorly water-soluble drugs was enhanced through cyclodextrins complexation. They also specified that medium to long chain fatty acids such as capric, lauric and oleic acids (C_{10}-C_{18}) have been proven to increase the absorptivity of a series of hydrophilic and hydrophobic drugs by changing the cytoskeleton of the intestinal epithelial cells without noticeable cytotoxicity.

Pluronics® (non-ionic surfactants)are characterized by their negligible toxicity. It can be utilized as solubilizers and wetting agent. It is well documented that Pluronics® may increase the absorption of many drugs(Brüsewitz et al., 2007).

PVP is the most frequently studied hydrophilic polymeric carrier as an absorption enhancer. It improves the dissolution of many drugs by improving their solubility due to its presence in the amorphous state (Kelvin's law), increasing the surface area subjected to dissolution due to size reduction of NPs. PVP is also considered as wetting agent; decreasing the interfacial tension between the drug and the aqueous medium(Gurunath et al., 2014).

D-α tocopheryl polyethylene glycol 1000 succinate (TPGS)is a water-soluble derivative of natural vitamin E, and it contains both a hydrophilic moiety and a lipophilic moiety, and therefore it is similar to conventional surfactants. TPGS is also a p-glycoprotein efflux inhibitor (Collnot et al., 2006).Accordingly, it has been effectively applied to advance NPs properties for controlled delivery. Mu and Feng (Mu and Feng, 2002)have applied TPGS as a surfactant stabilizer for the first time to fabricate paclitaxel-loaded PLGA nanospheres using solvent evaporation/extraction. They indicated that TPGS could be an ideal and effective emulsifier in NPs formulation. McCall and coworker(McCall and Sirianni, 2013)have applied TPGS as an emulsifier for the preparation of meloxicam loaded PLGA NPs.

Glycyrrhizic acid (GA) is available as a natural product. It is used in peptic ulcer treatment and as an expectorant (Tanaka et al., 1992, Motlekar et al., 2006, Anand et al., 2010). GA can be act as surfactant which is similar to bile acids (Motlekar et al., 2006). GA has been used to enhance the oral absorption of many drugs such as heparin (Motlekar et al., 2006). AmB rectal administration was improved significantly after GA addition to its formulation (Tanaka et al., 1992).

These listed above absorption enhancers are also considered as emulsifying agents or emulsifiers for formulations that could alter the characteristics of the resulting NPs. During the preparation process emulsifiers also stabilize the produced emulsion, decrease particle aggregation and affect the particle size and distribution, morphological properties, encapsulation efficiency and drug release characteristics of NPs(Coombes et al., 1998, Mu and Feng, 2002, Mitra and Lin, 2003). PVP is the most commonly used emulsifier during

the formulation of poly PLGA NPs because this emulsifier generates particles that are relatively uniform, small and easy to redisperse in aqueous medium(Sahoo et al., 2002).

Oral dosing is generally considered to be the most patient approachable and convenient route of drug administration. However, many pharmacologically active compounds cannot be administered orally because of inadequate oral bioavailability, and this may limit the usefulness of these compounds especially if the drug is subjected to intensive first pass effect. Poor oral bioavailability can be caused by poor aqueous solubility, degradation within the gastrointestinal contents, poor membrane permeability, or significant presystemic metabolism(Aungst, 2012).

In addition to absorption enhancers; cosolvency, pH adjustment and surfactant addition are the most commonly encountered pharmaceutical approaches for solubilizing drug with low aqueous solubility. Cosolvency (solvent blending) are mixtures of miscible solvents often added together which can be dramatically change the solubility of poorly aqueous soluble drugs, through reducing the interfacial tension between the aqueous solution and hydrophobic solute (Nayak and Panigrahi, 2012). The most commonly used cosolvents in pharmaceutical industry are ethanol, propylene glycol, glycerin, acetone, polyethylene glycols and dichloromethane(Jouyban, 2008). The combination of acetone dichloromethane as cosolvency has been used extensively to solubilize poorly soluble drugs (Aragón et al., 2010, Paudel and Van den Mooter, 2012).

1.5. Amphotericin B

Amphotericin B (AmB) is a polyene antibiotic, which was first isolated in 1959 from fermenter cultures of Streptomyces nodosus (Dutcher, 1968). The chemical structure of AmB ($C_{47}H_{73}NO_{17}$) is depicted in Figure 1.25(Lemke et al., 2005), AmB is amphiphilic. The rectangular ring structure is bipolar in nature, at one side is a hydrophobic trans conjugated heptane from C1 to C15 and at the other side is a highly hydroxylated hydrophilic side (C20 to C33). The structure also contains a carboxyl group (C60) and a primary amine (C48) and these have a pKa of 5.5 and 10, respectively, making AmB zwitter ionic at physiological pH. AmB is characterized by a large38-membered lactone ring linked covalent to an amino sugar moiety. This ring itself is made up of two chains.

The chain that contains the polyene chromophore is completely hydrophobic, whereas the chain that contains the hydroxyl group has a hydrophilic face and a hydrophobic phase, rendering it amphiphilic. The asymmetrical distribution of hydrophobic and hydrophilic groups for AmB confers an exceptionally low solubility for the drug in water and in many organic solvents as shown in Table 1.4(Lemke et al., 2005), which is one of the key factors in limiting its oral bioavailability and therapeutic applications. AmB is unstable at acidic pH of stomach. The high molecular weight (924.08 Da) and large cyclic structure of AmB account for the low intestinal permeability of the molecule (Asher and Schwartzman, 1977, Milhaud et al., 2002, Lemke et al., 2005, Jain and Kumar, 2010). AmB becomes extremely critical molecule since it has high molecular weight and tendency to form toxic aggregates. There is evidence that in water AmB forms a mixture of water-soluble monomers and oligomers along with insoluble aggregates(Patel et al., 2013).

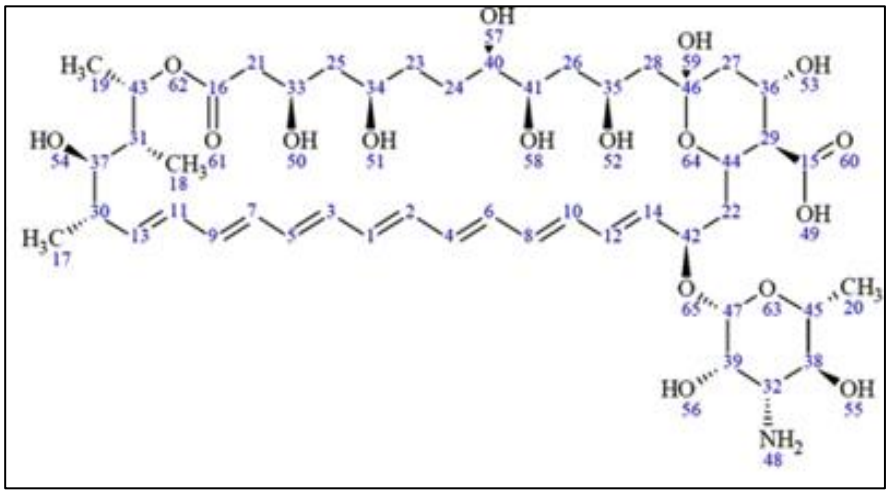


Figure 1.25. Chemical structure of Amphotericin (Lemke et al., 2005)

Table 1.4. Solubility of AmB in different solvents

Solvent	**Solubility (mg/l)**
Water	< 1 (at pH 6-7)
Methanol	2,000
Ethanol	500
Chloroform	100
Petrol ether	10
Dimethyl formamide	2,0
Propylene glycol	1,0
Cyclohexane	20

*(Lemke et al., 2005).

AmB is characterized by insolubility in water, but its water-solubility increases at a pH below 2 or above 11, where the drug being unstable and less antifungal activity as reported by Lemke et al., (Lemke et al., 2005). Fungizone® consist of AmB and sodium deoxycholate (solubilizing agent)(Lemke et al., 2005). In water, these two molecules (drug plus the additives) form micellar colloidal soluble complexes.

AmB is generally used for treating systemic fungal infections caused by species of *Candida, Aspergillus, Fusarium, Scedosporium,* and *Trichosporon*. Excellent reviews on fungal infections and their therapy have been written by several authors (Ellis, 2002, Herbrecht et al., 2003, Idemyor, 2003). AmB kills the fungal cells via primarily binding to ergosterol, which is the main sterol in the fungal cell membrane. These results in the destruction of osmotic integrity of the membrane resulting in the leakage of intracellular potassium, magnesium, sugars and metabolites thus inducing cell death. AmB has the widest antifungal spectrum and is used in clinical practice for almost all systemic fungal diseases. Most species of fungi that cause human infections are susceptible to AmB (Lemke et al., 2005).

The Biopharmaceutics classification system (BCS) is a systematic framework for categorizing a drug based on its solubility and intestinal permeability (Figure 1.26, (Amidon et al., 1995). Increasing the solubility of poorly soluble drugs can be achieved by modifying the shape, size, and functional groups present on the molecule, as well as increasing the permeability through the addition of lipid components into the drug. Loading drug(s) to NPs can altered the solubility and/or permeability of the entrapped drug (Paiv et al., 2013). This technique has been successfully used for drugs that belong to class II (low solubility-high permeability)such as Nifidipine (Kim et al., 1997), class III (high solubility-low permeability) such as Acyclovir (Giannavola et al., 2003), or class IV (low solubility-low permeability) such as Paclitaxel (Danhier et al., 2009). Drug loaded to NPs has significantly increased bioavailability compared to conventional form of the drug.

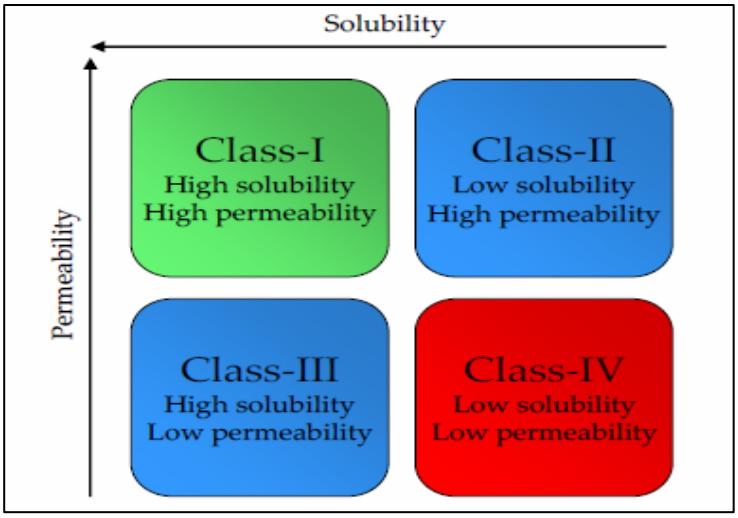


Figure1.26. The Biopharmaceutics Classification System (Amidon et al., 1995)

AmB belongs to class IV BCS and it is commercially available as Fungizone® (Bristol– Myers–Squibb), which is micellar suspension of 50 mg of AmB and 41 mg of sodium deoxycholate available as ivsince1960. Despite great effectiveness against broad spectrum of fungi strains, the use of Fungizone® is restricted due to severe side effects associated with its administration (Allémann et al., 1993, Walsh et al., 1995). Mainly, the AmB deoxycholate-related toxicity may be classified as acute or chronic. Acute or infusion-related toxicity is characterized by fever, chills, rigors, malaise, generalized aches, nausea, vomiting, and headache. Hypertension, hypotension, hypothermia, and bradycardia are other reported infusion-related toxic effects of administration of AmB deoxycholate (Chunn et al., 1977, Janknegt et al., 1996, Deray, 2002). Furthermore, thrombophlebitis may occur at the site of infusion due to slow infusion, central venous administration, and use of small-bore needles. Ventricular arrhythmias have been associated with administration of AmB deoxycholate in the context of hypokalemia, rapid infusion, anuria, and renal failure. A direct cardio-toxic effect has also been postulated. Nephrotoxicity is the most serious chronic adverse effect of AmB deoxycholate because the serum creatinine concentration increases in more than 80% of patients receiving this drug (Sabra and Branch, 1990). AmB-associated nephrotoxicity can be classified as glomerular or tubular. The clinical and laboratory manifestations of glomerular toxicity include a decrease in glomerular filtration rate and renal blood flow. While tubular toxicity is manifest as the presence of urinary casts, hypokalemia, hypomagnesemia, renal tubular acidosis, and

nephrocalcinosis (Burgess and Birchall, 1972). Although the exact mechanisms involved in AmB-induced azotemia have not been clearly described, investigators have established that amphotericin B can cause changes in tubular cell permeability to ions both *in vivo* and *in vitro*(Walsh et al., 1995). The azotemia may be due to tubuloglomerular feedback mechanism where increased delivery and reabsorption of chloride ions in the distal tubule initiate a decrease in the glomerular filtration rate of that nephron (Branch, 1988). Tubuloglomerular feedback is amplified by sodium deficiency. Other possible mechanisms for AmB-related nephrotoxicity include renal arteriolar spasm, calcium depletion during periods of ischemia, and direct tubular or renal cellular toxicity (Burgess and Birchall, 1972). Direct vasoconstriction caused in afferent arteriole by AmB can be another cause of nephrotoxicity (Sawaya et al., 1991). The actual mechanism is likely a combination of the above events.

There have been tremendous attempts to circumvent the poor water solubility and toxic side effects of AmB by synthesizing new derivatives or preparing novel carriers for AmB. This had led to the development of three lipid-based formulations in 1990 (Abelcet®, AmBisome® and Amphocil®) as shown in Table 1.5(Robinson and Nahata, 1999). They offer shorter course of therapy and are claimed to be more effective with lower toxicity compared to Fungizone® (Bekersky et al., 1999).

Table 1.5. Lipid formulations of amphotericin B general characteristics and monthly cost comparison

Trade name	FDA approval	AmB :Lipid ratio	Composition	Dosage mg	Cost** US$
Fungizone® Squibb Pharmaceutical	1958	-	Colloidal dispersion of AmB and sodium deoxycholate	50	7.5
Abelcet®Elan Pharmaceuticals	1995	1:3	AmB with phosphatidyl choline "ribbon structure "	100	241
Amphocil® Sequus Pharmaceuticals	1996	1:1	Colloidal dispersion AmB with cholesteryl sulfate	50 and 100	90-180
AmBisome® Nextar Pharmaceuticals	1997	1:9	AmB phospholipid liposome	50	189

**Prices represent cost per unit specified, are representative of average wholesale price.

Severe adverse reactions, such as anaphylaxis, cardiactoxicity, and respiratory failure, following administration of all three lipid formulations of AmB have been reported. In most reported cases, switching to a different lipid formulation of AmB was well tolerated (Burgess and Birchall, 1972, Deray, 2002). Beside their adverse effects, they available only as expensive parenteral therapy and so, AmB-lipid formulations is failing to spread all the infected patients in many areas and death rate keeps rising.

AmB-loaded-polymers have been studied extensively to enhance the solubility and bioavailability of the drug after iv administration. Several PEG conjugates of AmB have been designed and have demonstrated reduced toxicity in comparison to conventional AmB *in vivo*. The first PEG-AmB conjugate contained two molecules of AmB attached to PEG (Conover et al., 2003) and in this conjugate there was a reduction in toxicity *in vivo*. A second PEG-AmB conjugate with a pH sensitive imine linkage was then investigated(Sedlák et al., 2007). The conjugate was designed for site-directed drug release at pHs is 5.5, either in the intracellular compartments or at sites of localized fungal infection. This conjugate was 5 times less toxic *in vivo* than AmB delivered as Fungizone®, with a maximum tolerated dose of 45 mg/kg, whilst maintaining *in vivo* antifungal effectiveness against *Candida albicans*.

The oral route of administration remains the most convenient and widely used means of systemic drug delivery. Physicochemical properties of the drug molecule and gastro-intestinal physiology play vital roles in the design of an oral drug delivery system. Low aqueous solubility and permeability are the prime reasons accountable for the poor oral bioavailability of AmB. Therefore, its encapsulation in a suitable drug carrier, which is capable of shielding unfavorable biopharmaceutical properties of the drug and enhances the intestinal uptake, presents a possible solution. The formulation must also improve the stability of the drug in the harsh gastric pH as well as protect it from enzymatic degradation in the gastro-intestinal tract. Effective release of AmB from the carriers in its active monomeric form after entering into the body is also the pre-requisite to achieve desired therapeutic effect. All these factors must be balanced to develop a successful oral formulation of AmB.

1.6. Oral Amphotericin B Delivery Systems

The poor bioavailability of AmB can be contributed to its current parenteral administration for the treatment of systemic fungal infection or visceral leishmaniasis.

To date, there is no oral AmB formulations have been marketed due to is poor solubility and permeability. Therefore, developing an oral AmB formulation is a viable means of improving patient access to treatment worldwide.

A massive number of literatures have been reported to improve the delivery of AmB; as seen in Table 1.6. However, limited researchers have been published on its oral delivery.

AmB is also unable to cross the mucosal barrier of the GI to blood stream. The critical need for an oral drug delivery of AmB has steered several endeavors found in the literature about the development of AmB oral delivery systems to improve its antifungal activity and toxicity. These include formulating AmB as nanosuspension (Kayser et al., 2003), as poly(lactide-co-glycolide) nanoparticles (NPs) employing vitamin E-TPGS as a stabilizer (Italia et al., 2009, Italia et al., 2011), as lipid-based oral formulation using Peceol (Sachs-Barrable et al., 2008) or as liquid antisolvent precipitation NP(Zu et al., 2014). AmB was loaded to Peceol and PEG-phospholipids (iCo-009).These lipid-based AmB formulations were made as a self-emulsifying drug delivery system (SEDD) using proprietary mixture of mono- and diglycerides with phospholipids. This system was reported to improve the aqueous solubility of AmB, improve bioavailability and also exhibit significant anti-fungal activity upon oral and parenteral administration (Risovic et al., 2003, Amarji et al., 2007, Gershkovich et al., 2009, Wasan et al., 2009a, Sivak et al., 2011).

Furthermore, AmB was formulated with carbon nanotubes (Wu et al., 2005, Prajapati et al., 2011a, Prajapati et al., 2011b), or with gelatin coated lipid NP(Jain et al., 2012).

Different tactics were also applied such as chitosan–EDTA conjugate (Singh et al., 2013), cubosomes lipid-based delivery (Yang et al., 2012, Yang et al., 2014), nano-emulsions (Wasan et al., 2009a), polymeric nanoparticles (Italia et al., 2012) and solid lipid nanoparticles (Zhang et al., 2013).

Table 1.6. Formulation strategies (classes) of AmB*

Class	Microorganisms	References
Liposomes	Leishmania spp	(Davidson et al., 1996) (Oliva et al., 1995) (Yardley and Croft, 2000)
	C. albicans	(Carrillo-Munoz et al., 1999)
Polymer particles		
Polylactide-co-glycolide	L. donovani	(Venier-Julienne and Benoit, 1996)
Polymethylacrylate		(Sen et al., 1998)
Polyethylenimindextran		(Tiyaboonchai et al., 2001)
Nanosuspensions	Leishmania spp	(Kayser et al., 2003)
Solid lipid nano-	L. donovani	(Kayser et al., 2003)
Cyclodextrins		(Just-Nübling, 1993)
Emulsions	L. donovani	(Lamothe, 2001)
Detergents		(O'Neil and Lapointe, 1997)
Micellar systems		(Ramos et al., 1994)
Cochleates	L. donovani	(Santangelo et al., 2000)
	C. albicans	(Santangelo et al., 2000)

*(Lemke et al., 2005)

The latest formulations were AmB liposomes containing ceramides (Skiba-Lahiani et al., 2015) and AmB encapsulated with chitosan derivative (Serrano et al., 2015). These oral drug deliveries were developed to enhance the solubility and gastrointestinal permeability of AmB, but none of them has been introduced to the market yetmarket (Heurtault et al., 2003, Wasan et al., 2009a, Thornton and Wasan, 2009, Ibrahim et al., 2012, Yang et al., 2012).

1.7. Objectives of the Study

Hypothesis; AmB currently available asiv solution for parenteral administration. Utilizing nanotechnology to load AmB to the commercially available biodegradable Amphiphilic Block Copolymer (PLGA-PEG; Resomer®) will enhance the oral delivery of AmB in terms of encapsulation, release pattern, improve bioavailability, antifungal activity and reduce toxicity. The obtained novel oral AmB-NPs stealth could provide a long-circulating drug reservoir of AmB from which the drug can be released into the vascular

compartment in continuous and controlled manner for optimum drug targeting and minimizing the toxic effects. This can provide an effective use of AmB while curtailing its toxicity.

The aim of the present study is to formulate a novel oral dosage form containing AmB using PLGA-PEG copolymer to achieve the following benefits

1. Formulate an oral dosage form to avoid painful administration parenteral of AmB.
2. Enhance the permeability of the drug due to its loading into the polymer
3. Improve the oral bioavailability of the drug.
4. Decrease the nephrotoxic potential of the drug.
5. Decrease the adverse side effects of the drug.
6. Increase or no change of the therapeutic value of the drug.

Therefore, the main objectives of this investigation can be highlighted as follows:

1. To optimize the formulation composition of AmB-NPs prepared by emulsification diffusion method utilizing main particle size reduction and drug loading as a parameters.
2. To study the influence of process variables/parameters on the formulation characteristics.
3. To characterize the different physicochemical properties of the prepared AmB-NPs dosage form.
4. To study the surface morphology of the prepared formulation through SEM and TEM.
5. To study the Fourier Transfer Infra-red (FTIR) spectrum and differential scanning calorimetry (DSC) of pure drug, polymer, excipients and their mixtures to study chemical interaction between the drug and the polymer or between the drug and the excipients.

6. To study *in vitro* drug release through the polymeric NPs, analysis of drug release data by different kinetic models like I) Zero order, II) First order, III) Higuchi matrix, and IV) Korsmeyer and Peppas.

8. To develop analytical method for *in vitro* and *in vivo* analysis.

9. To conduct a comparative oral bioavailability study of the developed nanoparticle dosage form with a marketed formulation in animal model.

10. To carry out an *in vivo* nephrotoxicity study of the developed formulation in order.

11. To determine the nephrotoxic potential.

12. To determine the *in vitro* antileishmanial activity of the developed nanoparticle formulation containing amphotericin B.

Chapter II. Oral Formulations of Amphotericin-B Nanoparticles and their *In Vitro* Characterization

2.1. Introduction

The rate of opportunistic systemic fungal infections is increasing unavoidably, particularly in patients with cancer, recipients of organ transplants, diabetes and with congenital and acquired immune-deficiencies (Italia et al., 2011). In severely immune-compromised patients, it may involve other organs, such as brain and sinus, or even cause disseminated infection (Upton et al., 2007).

AmB is the gold standard drug for the treatment of systemic fungal infections, since 1953 (Dutcher, 1968). Fungizone®, the first conventional iv dosage, is formulated as a deoxycholate-soluble salt of AmB. However, its AE can lead to nephrotoxicity in 30% of the patients after dosing (Torrado et al., 2008, Van de Ven et al., 2012).

The amphoteric nature of AmB is responsible for its poor solubility in both aqueous and organic solvents. As a class IV compound (Ménez et al., 2007), according to BCS, AmB is characterized with limited solubility and permeability properties and with its high molecular weight, 924 Da, resulting in low (0.3%) bioavailability if given orally(Ouellette et al., 2004, Italia et al., 2009, Patel et al., 2013, Manyes et al., 2014).

Different tactics have been investigated to reduce AmB associated nephrotoxicity and other AE during Fungizone® administration. These include saline loading, alternative day dosing and dose reduction; all procedures were with limited success. Even the recent generation of AmB lipid formulations (Abelcet®, Amphocil® and AmBisome®) with improved therapeutic indexes than Fungizone®, their high cost of these formulations (12 to 40 times-more expensive than Fungizone® Table 1.5), the need of hospitalization for parenteral administration and the acute AE have restricted their widespread use (Adler-Moore and Proffitt, 2008, Ibrahim et al., 2012).

For oral administration, the drug must be able to cross the gastrointestinal (GI) epithelium. AmB has intrinsic low solubility, low intestinal permeability and shows poor oral bioavailability (< 0.3%). Recently, the literature are full of several attempts of the development of AmB oral delivery systems which, claim effective antifungal activity and with minimum toxicity.

It should be mentioned that only two oral AmB formulations were submitted to the FDA for clinical trials. The first one was an oral cochleate formulation (Biooral AmB, BioDelivery Sciences, International, USA) and its phase I clinical trial has been completed (Wasan et al., 2009b).

The second one was consisted of three oral lipid based formulations of AmB, namely iCo-009, iCo-010 and iCo-011 to enhance the absorption and efficacy of AmB. The improved efficacy of oral AmB lipid-based formulations was due to the resulted better absorption of AmB (Wasan et al., 2009a, Gershkovich et al., 2010, Sivak et al., 2011). Both iCo-010 and iCo-011 contain lipid, Peceol, surfactant, Gelucire 44/14, and VIT-E "TPGS" as a co-surfactant. Both formulations could be considered as SEDDS and are capable of forming instant nano-size emulsion in the GI fluids(Ibrahim et al., 2012).

However, neither of these two formulations have any published further clinical trials results, phase II, and to date, it is unclear whether these formulations of AmB will lead to commercially available pharmaceuticals (Yang et al., 2012).

Recently, there is an increasing interest in utilizing polymeric biodegradable polymers either natural or synthetic to prepare PNPs formulations, to improve the delivery of drugs(Barratt, 2003, Ma et al., 2008). Polyesters are the most widely used polymers which include PLA, PGA, polycaprolactone (PCL) and their copolymers such as PLGA which have been approved by the FDA (Parveen and Sahoo, 2008).

The rapid elimination of the polymeric drug carrier from the blood stream through phagocytosis by the RES is the one of the major problems of parenteral drug delivery systems (Stolnik et al., 1995, Owens and Peppas, 2006). Therefore, PEGylating using PEG would avoid phagocytosis. PEG is approved for the internal use in human by the FDA (Luo et al., 2002).

AmB has been loaded to PEG alone(Greenwald et al., 2003, Sedlák et al., 2007), to PEG derivative (Poly(benzyl-L-aspartate)-b-PEG) (Bawarski et al., 2008), to lipoprotein derivative-PEG (Shao et al., 2010b), to PEG-phospholipid micelles (Vakil and Kwon, 2005), to lipid and phospholipids formulations (Wasan et al., 2009a) and to PEG-liposomes composed of phospholipids and cholesterol (Moribe et al., 1999).

AmB loaded to PLGA alone (Sahoo et al., 2002, Sánchez-Brunete et al., 2005, Italia et al., 2009, Nahar and Jain, 2009, Souza et al., 2015, Yan et al., 2015), to PLGA and TPGS (Tang et al., 2014)to a combination of PLGA and lipid (Amaral et al., 2009, Gharib et al., 2011)and encapsulated in PLGA-dextran (Bang et al., 2008, Choi et al., 2008).

Amphiphilic block copolymers (ABCs) have the ability to form various types of NPs referred to as micelles, nanocapsules, nanospheres or core-corona NPs (Adams et al., 2003, Shuai et al., 2004). ABCs are biocompatible nano-containers in the size range of 10–100 nm characterized by a core-corona architecture in which the core serves as a reservoir for the incorporation of poorly water-soluble drugs, while the hydrophilic corona provides a protective interface between the core and the external medium (Gaucher et al., 2010).

Several studies reported AmB attached to ABC for parenteral administration such as AmB loaded to PEG-PLA(Jain and Kumar, 2010) AmB-(PLA-b-PEG) NPs coated with polysorbate 80 (Tween-80)(Ren et al., 2009), encapsulation of AmB in PEG-poly(ε-caprolactone)to form polymeric micelles (Vandermeulen et al., 2006). All previous studies showed significant increase bioavailability of AmB.

Furthermore, syntheses carboxylated PLGA-PEG copolymer encapsulated with AmB as a parenteral drug delivery of AmB using nano precipitation method (Kumar et al., 2015). As well as encapsulation of AmB with another performulated PLGA-PEG- NPs showed nanometric size (<200 nm) and to have low PDI (<0.162) and good entrapment efficiency (%DEE >53.0%) with enhanced antifungal activity (Nahar and Jain, 2009).

Paclitaxel loaded to pegylated PLGA NPs using a nanoprecipitation method, demonstrated enhanced NP delivery to prostate tumors as compared to equivalent non-targeted NPs (Cheng et al., 2007). Nanoparticles were prepared for etoposide-loaded PLGA-PEG using nanoprecipitation or single-emulsion solvent evaporation. Combining of TPGS and PVA led to increase in encapsulation efficiency (~90%) and significantly prolong duration of release and improved the bioavailability(Saadati and Dadashzadeh, 2014, Robbie, 1998).

Doxorubicin—PLGA–PEG micelles have been formulated and tested as a treatment for hepatocellular carcinoma. The drug-loaded micelles showed cytotoxicity on tumor cells

in vitro and *in vivo*. Application of the targeted micelles resulted in significant improvement in therapeutic response (Preacher et al., 2006).

The anti-tuberculosis drug Isoniazid (INH) loaded to PLGA-PEG-PLGA triblock polymer NPs have been prepared by sonication followed by a double emulsification technique. The drug loading efficiency significantly increased and uniform release for the drug for longer duration.

Accordingly, after literature review, these ABCs PLGA-PEG copolymer "PLGA-PEG copolymer® PEG" Copolymers), that have been shown to be biodegradable and biocompatible were employed in this study, as an AmB carrier for oral delivery formulations to enhance AmB solubility and bioavailability.

Optimization of AmB loaded to PLGA-PEG copolymer was achieved by utilizing several approaches including: polymer selection, cosolvency, pH adjustment, particle size reduction and addition of different surface active gents and absorption enhancer. The formulated AmB-NPs were subjected to *in vitro* analysis. Screening processes have included differential scanning calorimetry, particle size determination using zetasizer, surface morphology using scanning electron microscopy and transmission electron microscopy as well as Fourier Transform Infra-Red (FTIR) characterization. AmB-NP yield, drug loading and *in vitro* AmB release have been also studied.

2.2. Materials and Apparatus

2.2.1. Polymers and Chemicals

All material and chemicals are of analytical grade and used as received. Table 2.1 and 2.2 show the list of materials and polymers, used in this study and their manufacturer, respectively.

Table 2.1. Chemical used during formulation of AmB loaded nanoparticles

Chemical	Supplier	Address
Amphotericin B (99.8% purity)	Sigma–Aldrich	St. Louis, MO, USA
Nicardipine Hydrochloride (98% purity)		
Dimethylsulfoxide (DMSO)		
Triethylamin		
PLGA-PEG copolymers polymer	Boehringer Ingelheim	Ingelheim, Germany
Acetone	Fisher, Chemical	Fisher, UK
Dichloromethane		
Hydrochloric acid		
Methanol-HPLC grade		
Acetonitrile- HPLC grade		
Acetic acid		
Polyvinyl pyrrolidone (PVP)	BASF	Ludwigshafen, Germany
Miglyol®-812	Sasol	GmbH, Germany
Vitamin E (TPGS)	Peboc	Eastman, UK
Pluronic F68	Ruger Chemical	New Jersey, USA
Water was deionized and purified by a Milli-Q Reagent Grade water system	Millipore Corporation	Bedford, MX, USA
Formic acid (Methanoic acid)	Avonchem	UK

Table 2.2. Composition of the use PEGlyated PLGA-PEG copolymers types

Symbol	Commercial Name	Polymer type	Composition	Lactic to glycolic acid ratio	Content of PEG (%)	Molecular weight (Dalton)
A	RGP d 50105	Diblock	PLGA–PEG 6000	1:1	10	5000
B	Rgp t 50106	Triblock	PLGA–PEG 6000-PLGA	1:1	10	6000
C	RGP d 50155	Diblock	PLGA–PEG 6000	1:1	15	6000
D	RGP d 5055	Diblock	PLGA–PEG 6000	1:1	5	5000
E	R 203 H *	Monoblock	Poly(D,L-lactide)	1:1	-	24000-38000

*Non Peglyated PLGA-PEG copolymers.

2.2.2. Apparatus

Table 2.3 shows a list of the apparatus used in this study and the manufacturer.

Table 2.3. Apparatus used during the study

Apparatus	Manufacturer
Analytical balance Mettler Toledo-AG285 Sartorius –ME36S- MicroBalance	 Mettler, Greifensee, Switzerland -Sartorius AG, Goettingen, Germany
pH meter, Model 72	Beckman Instruments, Fullerton, USA
Thermostatically-controlled magnetic stirrer, SP46920, Cimarec® 2	Barnstead/Thermolyne, Iowa, USA
High speed stirrer (Ultra-Turrax T25)	IKA Labotecnik, Staufen, Germany
Oven, TV 10b	Memmert, Germany
Freeze dryer (Labconco-Free zone 4.5)	Kansas, US
Dissolution apparatus, ERWEKA DT 600 GmbH	Heusenstamm, Germany
Malvern Zetasizer	Nano ZS, Malvern Instruments, UK
Fourier Transform Infra-Red (FTIR) spectrophotometer	Perkin–Elmer-Spectrum 1600, Norwalk, USA
Scanning Electron microscopy (SEM, JEOL JSM-6060 LV,	Jeol Ltd., Tokyo, Japan
Transmission electron microscopy (TEM, JEM- 1011	
Differential scanning calorimeter (Perkin Elmer DSC7	Perkin Elmer Ltd., Norwalk, USA
Waters HPLC system equipped with a Waters 484 variable UV absorbance detector Waters 515 solvent delivery system and a Waters 717 plus autosampler	Waters, USA
300 µl glass autosampler vials presplit septum	
0.45 µm millipore filter	
Digital pH meter, Model 72, Phasar-1	Beckman Instruments, Fullerton, USA
Centrifuge, Biofuge Stratas	Heraeus, Japan
Vortex mixer	Whirlmixer-fission Scientific equipment, UK
Nitrogen Evaporator-OA-sys, multivap-118	Organomation Associates, INC, Berlin, Germany
1.8 ml Eppendorf tube	Eppendorf, Germany

2.3. Preparation of Amphotericin B loaded-Nanoparticles

This study employed the preparation of AmB-NPs by a modified emulsification-diffusion technique (Mora-Huertas et al., 2010). The material composition used in the study compared to the literature's are presented in Table 2.4. The schematic presentation for the preparation method is shown in Figure 2.1. The method requires the preparation of three phases: organic, aqueous and dilution phases. The organic phase was prepared by dissolving 20 mg of AmB in 5 ml of the organic phase (Acetone/Dichloromethane; (ACN/DCM: 6/1, v/v)) containing aliquot of PLGA-PEG copolymer and 200 µl of 5N HCl. The composition of the studied AmB-NPs formulations is listed in Table 2.5.

Table 2.4. Composition comparison of the material used in this study and the literature of the emulsion–diffusion method*

Material	Literature composition	This study composition
Active substance, mg	10–50	AmB 20–40
Percentage of Polymer in the inner phase	1.0–2.0	4.0
Percentage of Oil in inner phase	2.5–5.0	2.5
Inner phase solvent, ml	10.0	5.0
Percentage of Stabilizer and surfactant agents	2.0–5.0	1-4% PVP, 0.5% F-68 and 5% TPGS
External phase solvent, ml water	40	30
Dilution phase, ml water	200	200

* (Mora-Huertas et al., 2010)

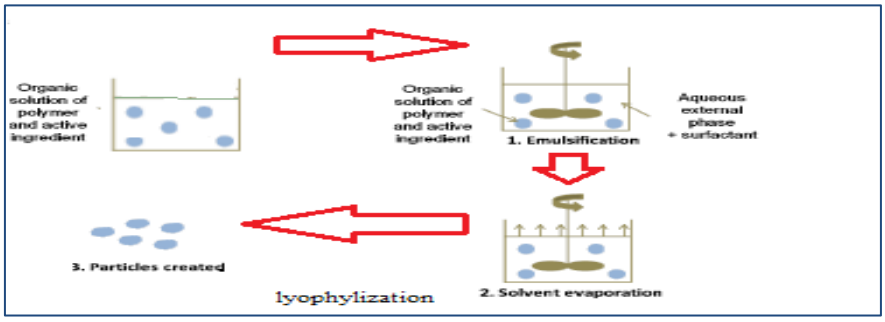


Figure 2.1. Set-up used for preparation of NPs by the emulsion–diffusion method

Table 2.5. Composition of amphotericin B- NPs produced by emulsion–diffusion method (n=6)

No.	Copolymer type	Phases					
		Aqueous phase [a]		Organic phase [b]		Dilution phase	Stirring Rate
		Drug (mg)	Miglyol (%)	PVA (%)	TPGS (%)	Water (ml)	rpm
A1	Diblock, PLGA–PEG 6000; Lactic to glycolic acid ratio (1:1) with 10%PEG , 5000 Dalton molecular weight (RGPd 50105)	20	---	1	---	200	8000
A2		20	---	1	---	200	13500
A3		20	---	1	---	200	24000
A4		20	2.5	1	---	200	24000
A5		20	2.5	1	5	200	24000
A6		20	2.5	4	5	200	24000
B3	Triblock, PLGA–PEG 6000-PLGA; Lactic to glycolic acid ratio (1:1) with 10% PEG , 5000 Dalton molecular weight (RGPt 50106)	20	---	1	---	200	24000
B4		20	2.5	1	---	200	24000
B5		20	2.5	1	5	200	24000
B6		20	2.5	4	5	200	24000
C3	Diblock PLGA–PEG 6000; Lactic to glycolic acid ratio (1:1) with 15%PEG , 6000 Dalton molecular weight (RGPd 50155)	20	---	1	---	200	24000
C4		20	2.5	1	---	200	24000
C5		20	2.5	1	5	200	24000
C6		20	2.5	4	5	200	24000
C7		40	2.5	4	5	200	24000
E	Monoblock Poly(D,L-lactide) (R 203 H)	40	2.5	4	5	200	24000
D3	Diblock PLGA–PEG 6000; Lactic to glycolic acid ratio (1:1) with 5% PEG , 5000 Dalton molecular weight RGPd 5055	20	---	1	---	200	24000
D4		20	2.5	1	---	200	24000
D5		20	2.5	1	5	200	24000
D6		20	2.5	4	5	200	24000

[a] Two hundred milligram of PLGA – PEG Copolymer and 200 µl of 5 NHCL were added to each formulation, except in case of C7-C8 500 µl was added.

[b] Addition of 0.15 gm of Pluronic-68 to all formulation.

All the vehicles used in this study were protected from light, either by using Amber containers or through covering containers with aluminum foil. Miglyol was added to the organic phase, then sonicated for 5 min and stirred until a clear solution was obtained using a magnetic stirrer. The aqueous phase was prepared by adding polyvinyl pyrrolidone (PVP), pluronic (F-68) and TPGS as stabilizers. The organic solution was added drop wise, over 5 min, to the aqueous phase using a high speed stirrer with Ultra-Turrax. The organic emulsion was added to 200 ml of distilled water to induce diffusion of the organic phase into the continuous phase, with mechanical stirring at 60^0C for 3 h. Each batch was prepared in six duplicate. All formulations were freeze-dried under vacuum (1.25 mBar) at -52 ^{0}C until complete dryness. Formulated dry NPs were stored in the freezer (-20 ^{0}C) prior to further investigation.

Four PLGA-PEG copolymers were investigated throughout the study, RGP d 50105 (A), RGP t 50106 (B), RGP d 50155 (C) and RGP d 5055 (D). Note, the letter indicates the copolymer used, while numbers were used to indicate the composition of the formulation; the same composition will have same number.

For optimizing the formulation process, the effect of each factor used on the AmB-NPs final formulations was investigated. The stirrer speed (8000, 13500, 24000 rpm) effect on four different PLGA-PEG copolymers prepared NPs were studied. The effect of PVP miglyol®-812 and TPGS concentrations on the final formulations were also studied.

Furthermore, in this study polymer R 203 H (poly(D,L-lactide))was included as formulation (E) to assess the effect of PEGylated copolymer.

Usually lyophilization of NPs required addition of cryo-protectant or lyo-protectant to be added to the formulation just prior to lyophilization. Typical lyo-protectant are listed in Table 2.6. Lyophilization of pure AmB required the addition of sucrose 10% before lyophilization to prevent aggregate formation. However, the formulated loaded AmB-PLGA-PEG copolymer NPs formulations were lyophilized without the addition of cryo-protectant during this study. The NPs formulations could be resuspended freely in aqueous solution after lyophilization. It was published that poly (ε-caprolactone) nanocapsules prepared with 2.5 or 5% of PVA could be freeze-dried without adding cryo or lyoprotectant (Abdelwahed et al., 2006, Singh et al., 2014). In another study, formulation of poly

(isobutylcyanoacrylate) and poly (isohexylcyanoacrylate) NPs could be freeze-dried without any modification of their size, in presence of 2% of pluronic®, without cryo protector (Abdelwahed et al., 2006, Balabathula et al., 2013).

During this study, AmB loaded to PLGA-PEG copolymer, formulations included the addition of PVP (1-4%) and pluronic (0.5%). These stabilizers could act as cryo-protectant.

Table 2.6. Examples of commonly used excipients in freeze-drying of pharmaceutical products*

Type	Function	Substance
Bulking agents	Offer bulk to the formulation particularly for very low concentration to freeze dried	Hydroxyethyl starch, trehalose, mannitol, lactose, and glycine.
Buffers	Regulate pH variations during freezing	Phosphate, tris HCl, citrate, and histidine
Stabilizers	Shield the product during freeze-drying against the freezing and the drying strains	Sucrose, lactose, glucose, trehalose, glycerol, mannitol, sorbitol, glycine, alanine, lysine, polyethylene glycol, dextran, and PVP.
Tonicity adjusters	Yield an isotonic solution and control osmotic pressure	Mannitol, sucrose, glycine, glycerol, and sodium chloride
Collapse temperature modifiers	Increase collapse temperature of the product to get higher drying temperatures	Dextran, hydroxypropyl-β-cyclodextrin, PEG, poly (vinyl pyro-lidone)

* (Abdelwahed et al., 2006)

2.4. Physicochemical Characterization of the Nanoparticles

2.4.1. Particle Size Analysis and Polydispersity Index (PDI)

The cumulative mean of particle size and polydispersity index (PDI) were determined by dynamic light scattering using zetasizer. Typically, after freeze drying of formulated formulation of AmB-NPs, the dried powder samples were suspended in deionized water and sonicated before measurement. The obtained homogeneous colloid was examined to determine the mean diameter, size distribution and polydispersity index. Viscosity and the refractive index of the continuous phase were set to those specific to water.

2.4.2. Zeta Potential Measurement

Zeta potential (ZP), which reflects the electric charge on the particle surface, is a useful parameter to calculate the physical stability of colloidal systems. ZP was determined by a Malvern Zetasizer. Diluted samples of 1.0 mg were diluted with Milli-Q® water. A fresh disposable folded capillary cells was used each time for measurement.

An average of 100 measurements at 25^0 C with prior equilibrate on of 1.0min was taken as a record.

2.4.3. Fourier Transform Infra-Red Spectroscopy (FTIR)

FTIR spectra of each pure component as well as the prepared freeze-dried AmB-NPs formulations were analyzed using a FTIR spectrophotometer. The measurements were performed from 450 to 4000 cm^{-1} at constant rate of 10 ^{0}C/min under an argon purge. AmB samples were diluted with KBr powder. The IR spectra were obtained in a KBr disc at ambient temperature.

2.4.4. Morphology of the Nanoparticles

2.4.4.1. Scanning Electron Microscopy (SEM)

SEM was used to examine the surface characteristics of the formulated NPs. The dry samples of each prepared AmB-NPs formulation were mounted onto metal stubs using double-sided adhesive tape. The stubs were vacuum coated with gold using fine coat ion sputter under reduced pressure prior to examination by SEM. The accelerating voltage was kept constant at 15 KV under an argon atmosphere. The SEM images were done in research center-King Saud University- female section with technical support from them.

2.4.4.2. Transmission Electron Microscopy (TEM)

TEM was used to examine the morphology of each prepared AmB-NPs formulation. The aqueous (water) dispersion of the freeze-dried NPs was placed over a 400 mesh copper grid covered with carbon film. After 2min, excess solution was wicked away by touching the edge of the grid with a filter paper and negative staining of the sample was conducted by a drop of 1% alkaline phosphotungstic acid (PTA) for 1min. The excess stain was wicked with filter paper and grid was allowed to air dry before observations, then

loaded the dry samples to the TEM. The TEM images were done in research center-King Saud University- female section with technical support from them.

2.4.5. Differential Scanning Calorimetry (DSC)

Samples of AmB loaded NPs were scanned to determine the thermal properties of the AmB in its NP formulations. Approximately 2 mg samples were weighed and placed into standard aluminum pans, which, were hermetically sealed. An empty pan was used as a reference. The transition temperature (Tc) and enthalpy of transition (ΔH) were determined from the thermogram, generated by a DSC. The heating rate was 10^0C/min from 30 to 200^0C, with a closed-pan system under a stream of argon gas flow, after which the system was cooled down at the same rate from 200 to 30^0C. The apparatus was calibrated with indium 99.99%. In addition, DSC scans were also performed for each pure component of the formulations. All the samples were freeze-dried before the measurements. The DSC images were done in research center-King Saud University- female section with technical support from them.

2.5. Determination of Drug Entrapment Efficiency and Nanoparticles Yield

Drug entrapment efficiency (DEE) of AmB in the NPs was calculated by determining the amount of AmB entrapped in the NPs. An aliquot of 10 mg sample of the freeze-dried NPs was first dissolved in 5 ml of DCM: ACN (1:6, v/v), 200 µl of 5N HCl and the volume were made up to10 ml with methanol: water (75:25, v/v). An aliquot of 1.25 ml of the stock solution was mixed with 1ml of Nicardipine (IS, 10 µg/ml) and the final volume was 10ml with methanol: water (50:50, v/v). Transfer it into a glass autosampler vial with pre-slit septum where, 75 µl were injected into the HPLC instruments. This experiment was performed in triplicate. Absorbance at 382 nm of diluted stock solution was measured using a validated high performance liquid chromatography (HPLC) method and the AmB concentration was calculated as described below.

DEE (%w/w) =100*(Mass of recovered AmB-NPs / Initial mass of drug used in formulation)

Yield (% w/w) = (mass of AmB-NPs / total mass of polymer, excipient and drug added) x100

2.6. HPLC Assay for *In vitro* Study

A new sensitive method for AmB was developed in this study. It involveda simple procedure suitable for routine work. The concentration of AmB was measured using a Waters HPLC system. Waters 515 solvent delivery system was used for the gradient flow through a Novapak C18 column (3.9x150 mm) packed with 5 µm spherical particles. The mobile phase consisted of acetonitrile: methanol: acetic acid: triethylamine (40.5: 4.5: 0.2: 0.1 % v/v, respectively). The mobile phase was prepared on a daily basis during the study, filtered through 0.22 µm millipore filter and degassed under vacuum. The mobile phase flow rate was 1.2 ml/min and the run time was 5.0 min. The injection volume was 75µl and detection was at 382 nm. Data were analyzed with an Empower Pro Chromatography Manager Data Collection System. The HPLC system was operated at ambient temperature.

A stock solution containing 0.2 mg/ml of AmB was preparedby dissolving 2.0 mg of the AmB in 10 ml mixture of DMSO and methanol (1:9, v/v) and stored in 4.0 ml polypropylene vials at −20^{o}C, since AmB adsorbs to glass (Lee et al., 2001), and covered with aluminum foil to protect AmB from light.

A stock solution containing 0.5 mg/ml of Nicardipine in methanol was used as the internal standard (IS)and it was stored at −20^{o}C. A daily standard calibration curve (n=3) ranging from 0.25 to 5.0 µg/ml containg 10µg/ml of the IS was prepared to determine the unknown AmB concentration for DEE and drug release. The standards were transferred to plastic autosampler vials with pre-slit septum (Waters, USA), where 75 µl were injected into the HPLC system for analysis.

2.6.1. Assay Validation

The developed method was validated in regards to linearity, precision, accuracy, according to ICH Harmonized Tripartite Guidelines(Guideline, 2005).

2.6.2. Assessment of Linearity, Accuracy and Precision

Three standard calibration lines were prepared in different times (at least three months) to evaluate the linearity, precision, accuracy and stability. The linearity of each standard curve was assessed by plotting the peak area ratio of AmB to IS versus AmB concentration.

Accuracy of the process stated in term of bias (percentage deviation from the nominal concentration= [100*(actual concentration-nominal concentration)/ actual concentration].The accuracy was assessed by examination of multiple replicates (n = 6) of AmB concentration.

Precision of a measurable technique is the degree of agreement among individual tests, when the technique is applied repetitively to analyze multiple replicates in three different occasions. The intraday precision was assessed by analysis of the calibration curves of 6 replicates of different concentrations of AmB within the same day. The inter-day precision was determined by the analysis of 6 replicates of different concentrations of AmB in 3 different days. The total precision of the method expressed as the relative standard deviation (RSD%).

Low, medium and high concentration quality control (QC) samples at concentrations of 0.25, 1.0 and 5.0 µg/ml of AmB with 10µg/ml of IS were analyzed, in three different occasions within at least three months, as described above.

The limits of detection (LOD) and of quantification (LOQ) were measured based on the analysis of 6 replicates. The LOQ was well-defined as the lowest drug concentration of the calibration daily curve which can be calculated with an accuracy <15% and precision < 12%. The LOD was defined as 0.5 times of the limit of quantification. In addition, the LOD presents a peak signal to noise of baseline ratio equivalent to 3:1, while the LOQ shows a ratio of 10:1.

2.7. Drug Dissolution in Phosphate Buffer

The quantitative *in vitro* release test was performed using US Pharmacopeia XXXII dissolution apparatus 2 (paddle). The dissolution was carried out in 500 ml phosphate buffer containing 2% sodium deoxycholate (pH 6.8 ±0.1) at 50 rpm and the temperature was maintained at 37 ± 0.5 ^{o}C. A sample of AmB NPs equivalent to 5.0 mg of AmB was placed on the surface of the dissolution medium. At appropriate time intervals (0.25, 0.5, 1, 2, 3, 4, 5, 6 and 24 hours), 2.0 ml samples were withdrawn from each vessel, mixed with 1.0 ml of IS (10µg/ml) and made up to 5.0 ml with methanol: water (75:25, v/v). The solution was filtered through a 0.22 µm Millipore membrane filter, transferred to an autosampler vial with pre-slit septum (Waters, USA)and 75 µl was injected and analyzed

by HPLC. This experiment was done in triplicate. The withdrawn volume was replaced each time with 2 ml of fresh medium kept at 37 ± 0.5 ^{o}C to maintain a sink condition.

2.8. Drug Release Kinetics

Drug release kinetics of AmB formulations were analyzed using various dissolution models including; zero, first, Higuchi and Korsmeyer–Peppas order (Table 2.7). Release profile data were processed and plotted according to the equations of different models followed by regression analyses. The criterion for selecting the most appropriate model was based on best goodness-of-fit (R^2 values). The slope of each plot and its release rate constant for each particular model were used to describe the release rate mechanism.

Table 2.7. Drug release kinetics equation and comments

Model	Equation	The order
Zero order*	$Q_t = Q_0 - K_0 t$	This equation is for drugs with no aggregation in the dissolution media and the drug release is slowly: Q_t : The amount of drug released in time *t*, Q_0: The cumulative amount of drug released K_0: Zero order release rate constant
First order*	Ln Q_t = Ln Q_0 – Kt	Q_t and Q_0: As above. K: First order release constant
Higuchi model**	$Q_t = Kt^{1/2}$	Where Q_t is as above and K is the Higuchi rate constant
Korsmeyer-Peppas model***	$Q_t / Q_\infty = Kt^n$	Q_t / Q_∞: The fraction of drug released at time t, K: The release rate constant n: The release exponent. n< 0.5: Fickian diffusion; 0.5<n<1: Anomalous diffusion(non- Fickian transport),n=1: Zero-order release

*(Costa and Sousa Lobo, 2001) ** (Dash et al., 2010) *** (Shoaib et al., 2006)

2.8.1. Data and statistical analysis

In vitro results were expressed as mean ± SD of at least three replicates. The HPLC results of AmB were calculated using linear regression without weighting, according to the equation: Y= 0.379X -0.041, where, Y is the area under the peak (AUP) ratio of the drug to the internal standard and X is the concentration of AmB. The RSD % was calculated for all values. The Student's *t*-test was used to inspect the concentration difference at each day

and one-way analysis of variance (ANOVA) was used to assess the reproducibility of the assay and the drug dissolution from batch to batch using IBMSPSS Statistics 21. The level of confidence was 95%.

2.9. Results and Discussion of *in vitro* Characterization

2.9.1. Optimization of AmB-NPs Formulation Process

The choice of a specific method of NPs preparation is a factor of the physicochemical properties of the drug to be loaded as well as the intended route of drug administration. For hydrophobic drugs, nanoprecipitation (Barichello et al., 1999, Peltonen et al., 2004, Bilati et al., 2005, Vauthier and Bouchemal, 2009) or an emulsion–diffusion method are best suited for NPs preparation (Kwon et al., 2001, Soppimath et al., 2001, Hans and Lowman, 2002, Dillen et al., 2006, Italia et al., 2009, Mora-Huertas et al., 2010, Mora-Huertas et al., 2011).

Two steps are involved in emulsion diffusion evaporation method. The first step involves formation of primary emulsion with small droplet of organic solvent containing drug and polymer in aqueous phase (oil/water). In the second step, the emulsion was homogenized at high speed to decrease the particle size before the removal of the organic solvent by evaporation, resulting in formation of NPs. In contrast, the nanoprecipitation method involves a single step in the formation of NPs. The organic phase containing drug and polymer was added to the aqueous phase, resulting in rapid de-solvation of the polymer due to diffusion of solvent into the aqueous phase. This precipitation of polymer with the drug caused an instantaneous formation of NPs of the drug loaded to the polymer matrix.

The emulsification-diffusion method has been used successfully to prepare biodegradable NPs in an efficient and reproducible manner especially for hydrophobic drugs(Quintanar-Guerrero et al., 1996, Quintanar-Guerrero et al., 1998a, Kwon et al., 2001, Soppimath et al., 2001, Hans and Lowman, 2002, Galindo-Rodriguez et al., 2004, Dillen et al., 2006, Mora-Huertas et al., 2010, Mora-Huertas et al., 2011). Similar finding was observed with AmB in this study since it isa hydrophobic drug.

Selection of the proper organic solvent was the most challenging issue to overcome AmB poor solubility in organic solvent (Table 1.4). After acidification with 200 µl of 5N HCL, ACN/DCM (6:1, v/v) was found to be the most suitable cosolvents for AmB and the copolymer used during the study.

2.9.2. Factors Affecting Nanoparticles Formulations

2.9.2.1. Shearing Rate

Different speeds of mechanical stirring ranging from 8000 to 24000 rpm were tested in this study. The relationship between stirring speed and mean particle size (MPS) is shown in Table 2.8. Several publications correlate the stirring speed (mixing speed) with the MPS of NPs. They found that increasing the stirring speed, significantly reduces the size range of the NPs This range is controlled and considerably narrowed at higher stirring speed(Eldem et al., 2001, Brime et al., 2003, Balabathula et al., 2013, Ibrahim et al., 2013). The NPs size was inversely proportional to the stirring speed. A significant reduction in NPs size range (from 1068.1 ± 489.8 to 400.2 ± 62.1 nm) was observed on increasing the shearing rate from 8000 to 24,000 rpm for the "A" formulations. The same results were obtained with the other tested formulations of different polymers (B, C and D). At the lowest stirring speed (8000 rpm), the A1 showed the highest MPS (1,068.1 ± 489.8 nm) while C1 showed the lowest MPS at this speed. While, at the highest speed (24000 rpm) D3 formulation showed the highest MPS 515.6 ± 30.7 in comparison to C3 with the lowest MPS (57.2 ± 7.5 nm). It should be mentioned that at higher stirring speed, the NPs formulations were arranged in the following order C > D> B> A, according to MPS reduction. Therefore, throughout this study, the 24,000 rpm stirring rate was used for the NPs preparation.

Table 2.8. Influence of shearing rate on the mean particle size and poly dispersity index of AmB-NPs prepared by emulsification –diffusion method

Batch number	Particle size (nm) ± SD	Polydisper-sity Index ± SD	Stirring Speed rpm	DEE %	Yield%
A1	1,068.1 ± 489.8	0.46 ± 0.1	8,000	18.5±3.3	67.3± 2.1
A2	451.2 ± 84.2	0.46 ± 0.1	13,500	21.4± 3.4	73.7±1.2
A3	400.2 ± 62.1	0.64 ± 0.3	24,000	23.3± 7.3	75.0±1.4
B1	336.5 ± 60.4	0.40 ± 0.1	8,000	17.2 ± 2.3	70.1± 2.1
B2	257.1 ± 13.6	0.40 ± 0.1	13,500	18.4± 3.5	77.4±1.2
B3	107.4 ± 62.0	0.50 ± 0.2	24,000	20.4 ± 1.4	74.8 ±1.5
C1	274.2 ± 30.1	0.40 ± 0.1	8,000	21.5± 4.3	76.3± 2.1
C2	226.9 ± 17.2	0.40 ± 0.1	13,500	23.4± 3.4	79.4±1.2
C3	57.2 ± 7.5	0.30 ± 0.1	24,000	25.9 ± 0.5	82.5 ± 1.1
D1	630.9 ± 67.4	0.40 ± 0.1	8,000	18.5± 3.1	70.7± 2.1
D2	538.6 ± 41.9	0.40 ± 0.1	13,500	21.4± 2.9	74.3±1.2
D 3	515.6 ± 30.7	0.40 ± 0.1	24,000	24.3 ± 2.3	85.5 ± 1.4

2.9.2.2. Addition of Surfactant and Emulsifier

Several papers report that the addition of surfactant or emulsifier significantly reduces the MPS due to its adsorption on the surface of the NPs, preventing the NPs aggregation by static repulsion and special hindrance (Santra et al., 2001, Schubert and Müller-Goymann, 2005, Kvitek et al., 2008, Balabathula et al., 2013). However, other studies suggest that the addition of a combination of more than one surfactant or emulsifier is better choice to reduce the aggregation (Mu and Feng, 2002, Mei et al., 2009, Chaudhari et al., 2012, Saadati and Dadashzadeh, 2014). In this study, more than one surfactant was incorporated to increase the reduction of the MPS and improve the stability of the NPs. Therefore the addition of PVP; hydrophilic surfactant, pluronic F-68 non-ionic surfactants and TPGS were investigated.

The main basic constituents of AmB-NP formulations were AmB (20 mg), PVP (1%) and F-68 (0.5%). The different composition of NPs investigated during the study and

their effect on the DEE%, yield% and the MPS of the NPs were summarized in Tables 2.8 and 2.9. All the AmB-NP formulations have homogenous distribution with PDI of< 0.5, in the nanometer size range with narrow size distribution and without polymer flakes or visible oil droplets.

Table 2.9. Influence of different composition parameters on the mean particle size and poly dispersity index of AmB-NPs prepared by emulsification –diffusion method

Batch number	Particle size (nm) ± SD	poly dispersity Index ± SD	Zeta Potential ± SD	DEE %	Yield%
A3	400.2 ± 62.1	0.64 ± 0.3	-21.9 ± 2.5	23.3± 7.3	75.0±1.4
A4	126.8 ± 27.3	0.53 ± 0.2	-24.8 ± 1.3	23.9 ± 1.1	81.3±2.2
A5	105.2 ± 9.3	0.26 ± 0.1	-24.0 ± 2.0	24.8 ± 4.1	82.5±1.3
A6	93.3 ± 5.7	0.51 ± 0.3	-23.7 ± 1.3	37.8 ± 7.5	83.7 ±1.4
B3	107.4 ± 61.9	0.53 ± 0.3	-22.5 ± 3.0	20.4 ± 1.4	74.8 ±1.5
B4	97.4 ± 10.2	0.39 ± 0.1	-25.0 ± 3.2	21.2± 7.1	77.1 ±2.3
B5	69.4 ± 14.7	0.24 ± 0.1	-25.4 ± 4.1	36.9 ± 2.4	78.9 ±2.4
B6	55.4 ± 5.9	0.26 ± 0.1	-25.3 ± 5.3	37.5± 1.7	80.9 ± 1.7
C3	57.2 ± 7.5	0.25 ± 0.1	-25.2 ± 3.5	25.9 ± 0.5	82.5 ± 1.1
C4	36.8 ± 7.6	0.28 ± 0.1	-30.2 ± 3.3	29.9 ± 6.2	83.8 ± 1.2
C5	27.0 ± 5.6	0.34 ± 0.1	-28.5 ± 1.2	40.6 ± 10.9	87.5 ± 1.1
C6	23.8 ± 4.8	0.31 ± 0.0	-29.9 ± 1.7	48.3 ± 4.2	90.6 ± 0.5
C7	25.3± 2.7	0.31 ± 0.0	-29.8 ± 2.1	56.5 ± 3.9	93.2 ± 0.5
E	539.9± 51.1	0.35 ± 0.1	-23.3 ± 3.5	27.2 ± 3.2	67.4 ± 0.8
D3	515.6 ± 30.7	0.37 ± 0.0	-23.9 ± 1.7	24.3 ± 2.3	85.5 ± 1.4
D4	418.8 ± 28.2	0.32 ± 0.1	-25.1 ± 2.4	24.4 ± 2.7	86.9 ± 2.2
D5	344.4 ± 38.4	0.26 ± 0.10	-25.5 ± 2.7	27.4 ± 2.5	88.1 ± 1.7
D6	318.4 ± 36.8	0.36 ± 0.10	-25.8 ± 2.8	39.4 ± 9.2	89.6 ± 1.0

A: RGPd 50105, B: RGPt 50106, C: RGPd 50155, D: RGPd 5055, E: R 203 H polymer

The size of different formulations were observed after varying one parameter at a timeand keeping other parameters constant. Selection of the particularparameter out of the set was based on; smaller size,size distribution (PDI-polydispersity index), and highest yield of drug loaded to the polymer used.

Among the tested basic component formulations, AmB loaded to copolymer C has the lowest MPS. On the other hand, AmB loaded to copolymer D has the highest MPS as shown in Table 2.9.

A significant reduction in MPS ($P<0.05$) was observed after the addition of miglyol-812 (2.5%) to the basic component of each copolymer (Formulations A4, B4, C4, D4). The size reduction was in the order of A (68 %) > C (35%) > D (18.9%) > B (8.5%) (Table 2.9). Therefore, miglyol-812 (2.5%) was added to the basic composition of the AmB-NPs starting from formulations with number 5.

The addition of the TPGS (5% w/v) causing further reduction in MPS of A (16.6%), D (17.9%), B (27.8%) and C (27%) (Table 2.9). Therefore, TPGS was also added to the basic component of the AmB-NPs formulation starting from formulations with number 6.

Moreover, increasing the concentration of PVP from 1% to 4%, the MPS of AmB-NPs formulation was also decreased in the order B, A, C, D (21.5%, 11.5 %, 11.1%, and 7.5% respectively) as shown in Table 2.9.

Interestingly, changing the PLGA-PEG copolymer to R 203 H polymer (non-peglyated) formulation E, caused a significant increase in the MPS > 95%. It should be mentioned that no more than 40 mg of AmB could be incorporated in these formulations (C7), with no significant effect on MPS. Therefore, formulations A6, B6, C6, C7, E and D6 with the lowest MPS were selected for *in vitro* characterizations.

Particle size of drug-loaded NPs is an important determinant affecting the *in vitro* drug release as well as its *in vivo* performance (Kim et al., 1998, Couvreur and Vauthier, 2006, Jin et al., 2007, Xin et al., 2010, Pham et al., 2013).

AmB –PEG nanoparticles formulations showed reduce MPS and improve drug release profiles associated with no toxic effect as compared with conventional solution formulation, Fungizone®(Tiyaboonchai et al., 2001, Kayser et al., 2003, Angra et al., 2009).

The size of AmB-NPs was determined by dynamic light scattering (NanoZS, Malvern Instruments, UK) as an average of 3 measurements.Measurment of the particle size is done through dynamic light scattering technique (Cegnar et al., 2004, Xu et al., 2006). Since suspended particles in solution move in Brownian motion, when subject to irradiation with a monochromatic laser there will be fluctuations in the intensity of scattered light. The autocorrelation function was then processed to determine the diffusion coefficient from which the average radius can be determined (Böschel et al., 2003, Vega et al., 2003).

2.9.3. Drug Encapsulation Efficiency and Nanoparticles Yield

DEE of the AmB NPs was found to be inversely proportional to the MPS (Table 2.9). The minimum DEE for all formulated formulation was 20% (B3) while the maximum encapsulation was 56.5 % (C7). Meanwhile, for the selected formulations the DEE was in the following order C7>C6> D6> A6 > B6 corresponding to 56.5 %, 48 %, 39 %, 37% and 32 %, respectively. The non-peglyated polymer (E) showed a DEE of 27 %. Howeverو the highest yield of AmB-NPs was 93% (C7) while the lowest one was 74% (B6).

2.9.4. Poly Disparity Index (PDI)

PDI values ranged from 0 to 1.0 PDI represents the particle size distribution within the formulations with values below 0.3 indicating a narrow size distribution and thus good long-term stability, while a higher value indicates a less homogenous NP size distribution. Tables 2.8 and 2.9 indicate that PDI in all formulations is < 0.5; which means that all formulations have homogenous and have narrow size distribution associated with good long-term stability in water. These findings are consistent with literature reports that NPs with cystatin incorporated in PLG-NPs (Cegnar et al., 2004, Xu et al., 2006).

Moreover, it is interesting to report that the addition of any surfactant or stabilizer to the basic composition of the NPs showed no trend of change in PDI value with no correlation between the MPS and the PDI values.

2.9.5. Zeta Potential Analysis of AmB- NPs Formulations

The change in electrical charge between the layers of ions around the NPs and that of the bulk of the surrounding fluid is known as zeta potential (ZP) (Chouhan and Bajpai, 2009). Several factors affecting the ZP measurement such as polymer composition(Tobıo et al., 2000), physicochemical characterization of the drug entrapped as well as stabilizer used during NPs formulation(Betancourt et al., 2007). The ZP is a measure of the degree of repulsion between similarity charged particles in the dispersion, thus, colloids with a high ZP (positive or negative) are electrically stable. It is reported that the stability of NPs mainly related to a high ZP (high surface charge), while low ZP values correlate NPs aggregates and instability (Betancourt et al., 2007, Feng et al., 2007).

Nanoparticles with ZP values greater than +25 mV or less than -25 mV typically lead to repulsive forces between the droplets which may improve the physical stability of the dispersion system, while system with a low ZP value will eventually aggregate due to Van Der Waal inter-particle attractions (Dobrovolskaia et al., 2009).

A negative surface charge was observed for all measured AmB loaded to PLGA-PEG NPs. Their ZP values are negative and in the range of -21.9 ± 2.5 to -30.2 ± 3.3 mV (Table 2.9). This finding was in agreement with other studies using polyesters polymers for NPs preparations such as paclitaxel loaded to PLA-TPGS and antiestrogen RU58668 loaded to PLGA-PEG, both show negative ZP measurements (Ameller et al., 2003, Xu et al., 2006, Pan and Feng, 2008).

It is interesting to report the addition of any surfactant or stabilizer to the basic composition of the NPs showed no trend of change in ZP value and there was no correlation between the MPS and the ZP values.

2.9.6. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) can produce high-resolution images of the specimen surface (in the nm range) and images can be viewed to determine their three dimensional appearance, thus showing the morphology of the specimen. SEM instruments

work by the measurement of electrons emitted from the surface after itis bombarded by an electron source.

The representative SEM analysis was also performed to complete the information about particle size and morphology. Figure 2.2 showed that formulation A6 (contain three surfactant) were the best spherical NPs with minimal aggregation in comparison to other formulations. Figure 2.3, formulations containing polymer B, indicate that polymer used did not improve the spherical structure of NPs than obtained when utilize polymer A in the formulation. The C polymer formulation (Figure 2.4) showed nanosized spherical with no aggregate NPs. Meanwhile Figure 2.5 although triblock polymer (D) were used showed nanosized spherical NPs with no aggregates. Figure 2.6, comparing formulations number 6 in all polymers used displayed that polymer E (non PEGlyated) showed the highest particle size in comparison to other polymers. While C6 followed by C7 were morphologically the best.

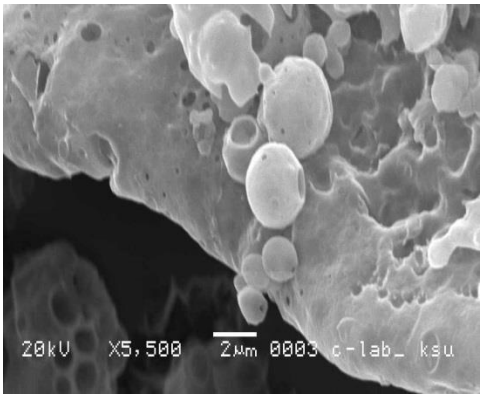
In conclusion, the SEM images (Figures 2.2-2.6) indicate that the presence of fairy spherical shape and discrete NPs with a variation in size distribution (in the nanometer size range) including both isolated and agglomerated particles. Figure 2.6, showed the SEM images for the selected formulation, which will be used for further *in vivo* study.

2.9.7. Transmission Electron Microscopy (TEM)

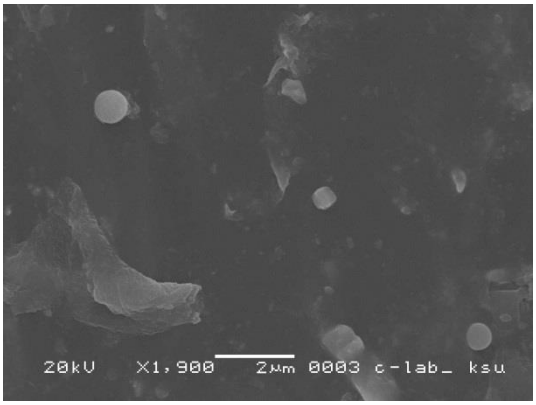
Transmission electron microscopy (TEM) is currently the technique most widely used to study nanomaterial morphology, providing a two-dimensional picture of these materials. The specimens for analysis were prepared by scraping few milligrams of a sample to form a thin film deposited on a glass microscope slide. The TEM analysis provided additional evidence regarding the spherical geometry of AmB-NPs formulations.

The TEM photographs showed that all AmB-NPs formulations were spherical in shape and in the nanometer size range (Figures 2.7-2.10). Furthermore, the surface morphology of these formulated AmB-NPs was found to be smooth with minimal aggregation. Formulation containing polymer C and B where the best in morphology and formulation number 6 is the best in all types of polymer use. Figure 2.11, is the TEM image of the selected formulation which will be used *in vivo* study.

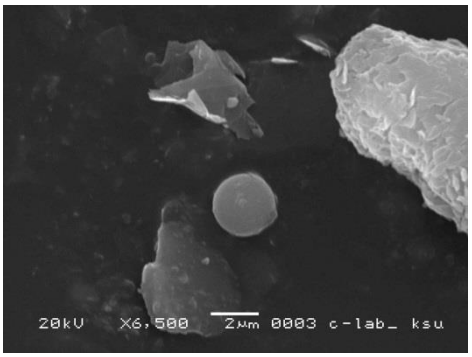
A_1



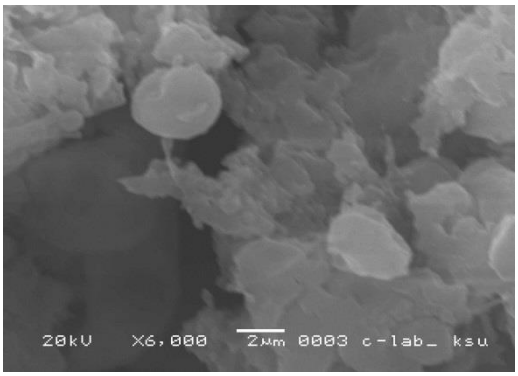
A_2



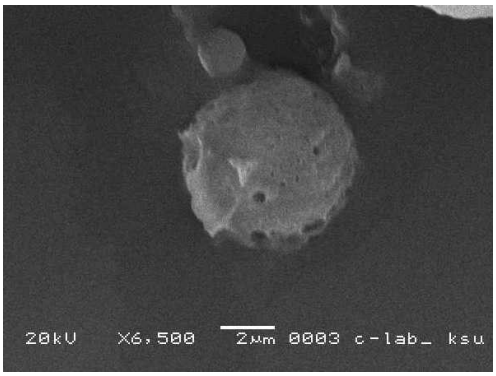
A_3



A_4



A_5



A_6

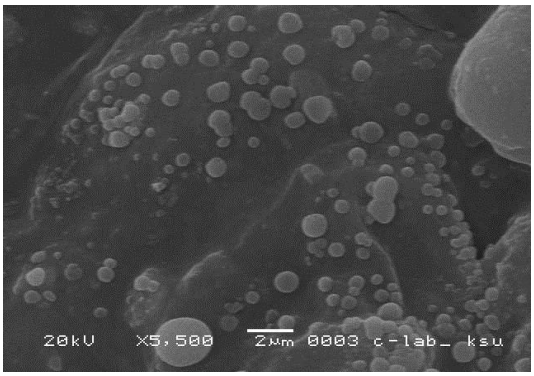
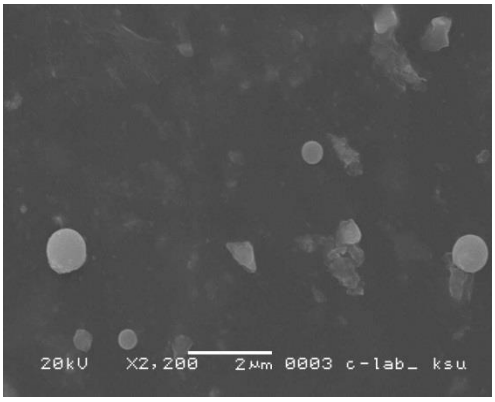
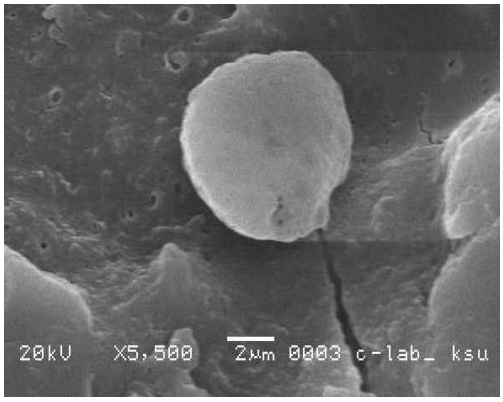


Figure 2.2.SEM images for AmB-NPs prepared by RGPd 50105 copolymer (A) formulations

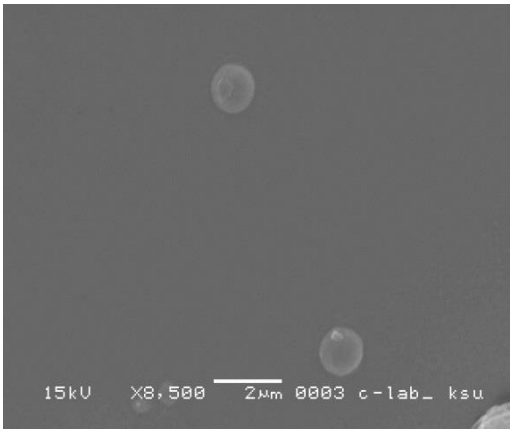
B_3



B_4



B_5



B_6

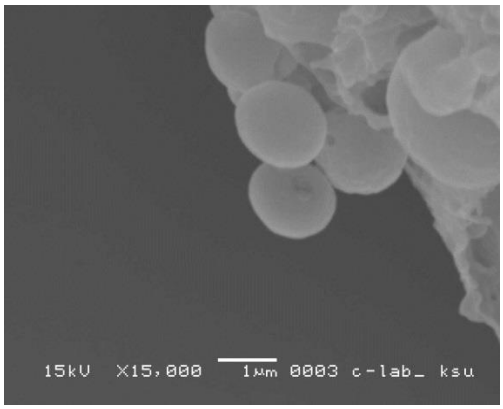
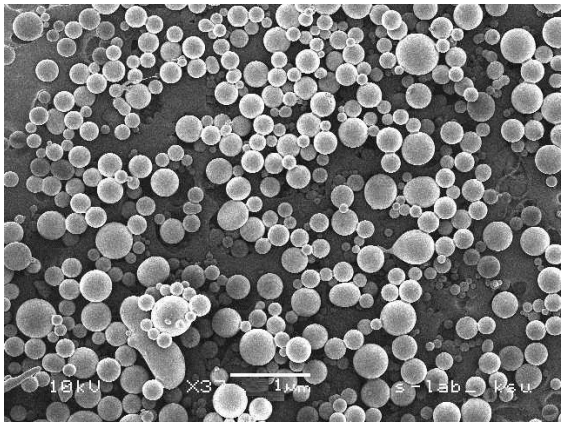
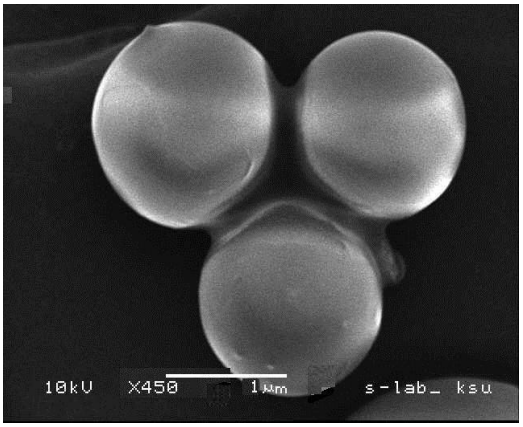


Figure 2.3.SEM images for AmB-NPs prepared by RGPt 50106 copolymer (B) formulations

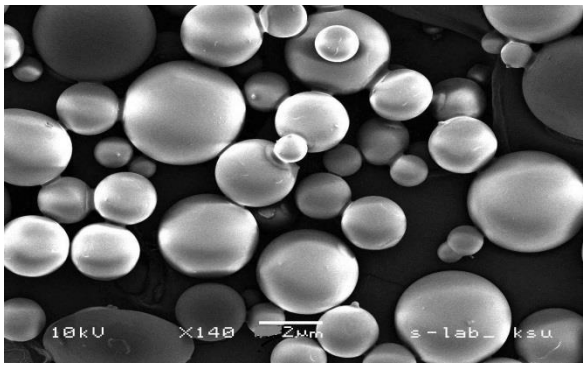
C_3



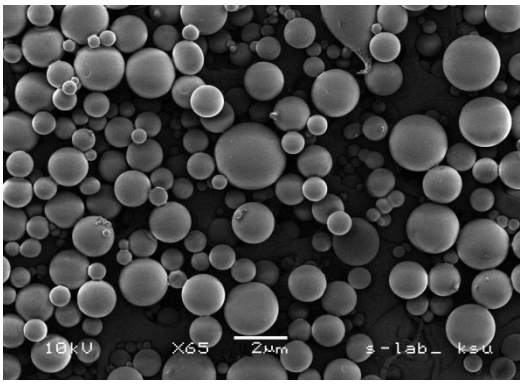
C_4



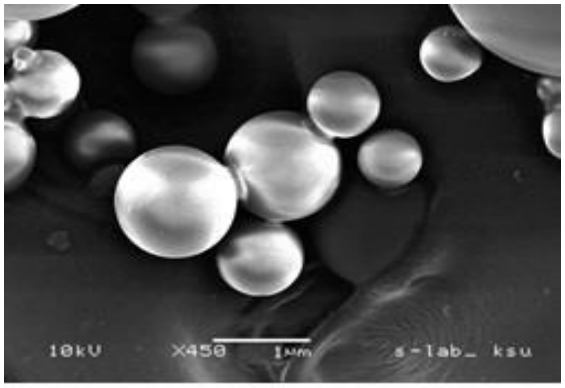
C_5



C_6



C7



E

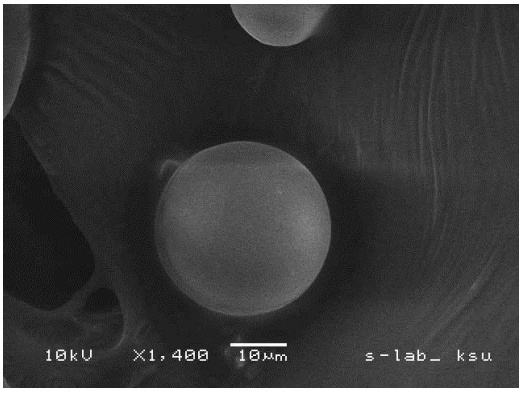
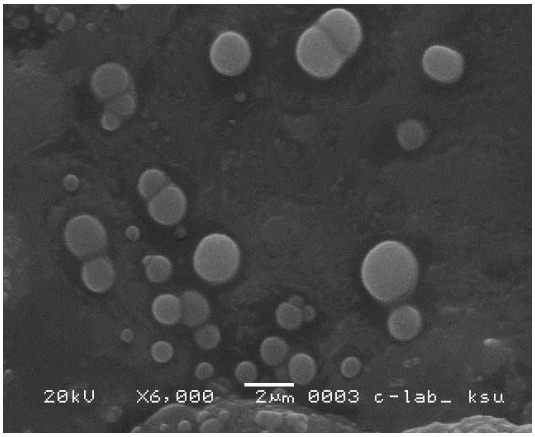
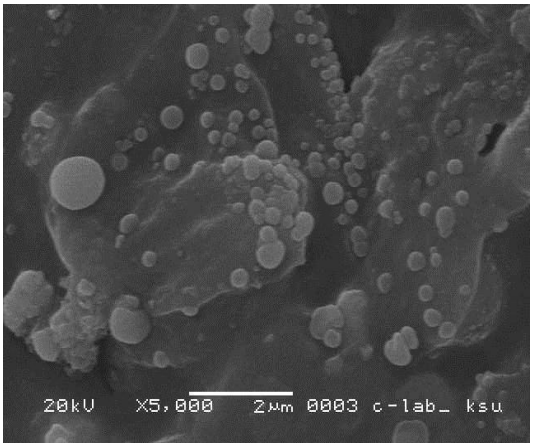


Figure 2.4. SEM images for AmB-NPs prepared by RGPd 50155 copolymer (C) and (E) with R 203 H polymer formulations

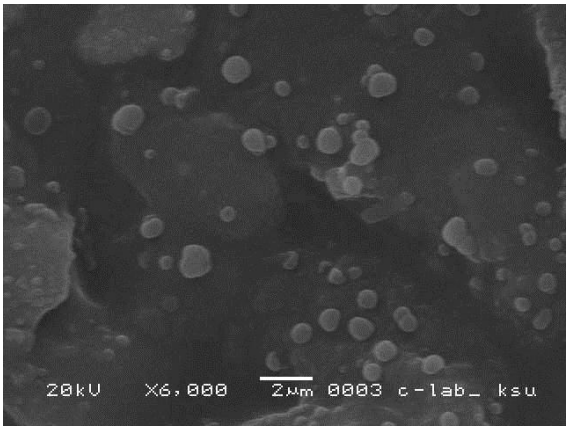
D_3



D_4



D_5



D_6

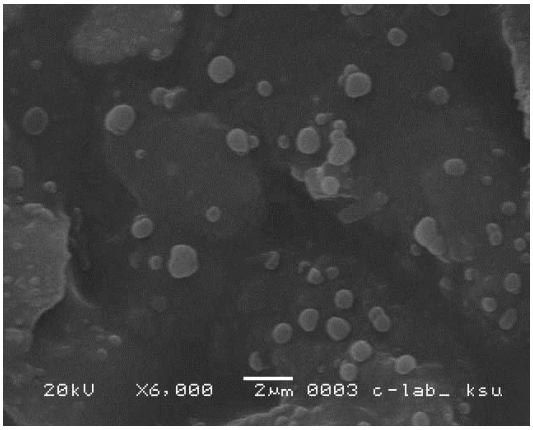
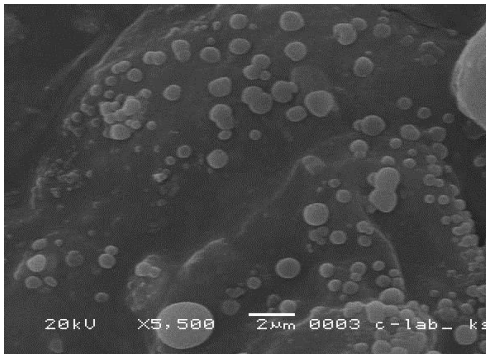
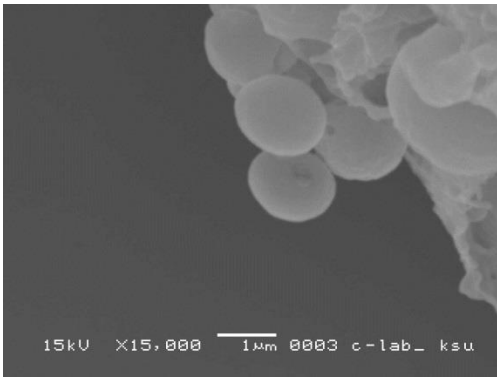


Figure 2.5.SEM images for AmB-NPs prepared by RGPd 5055 copolymer (D) formulations

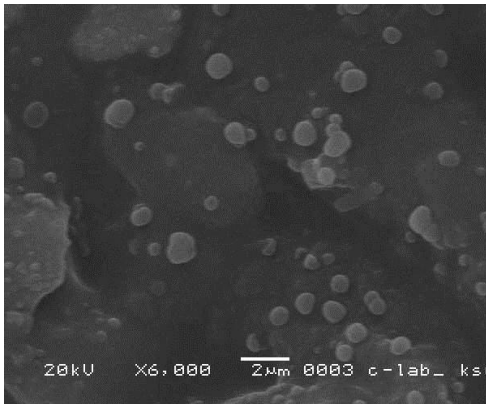
A_6



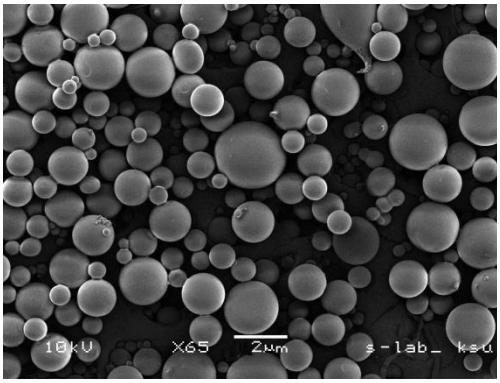
B_6



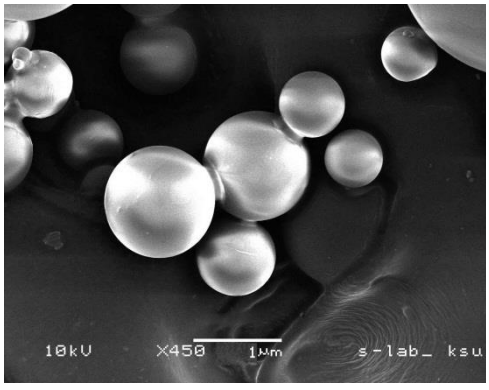
D6



C_6



C7



E

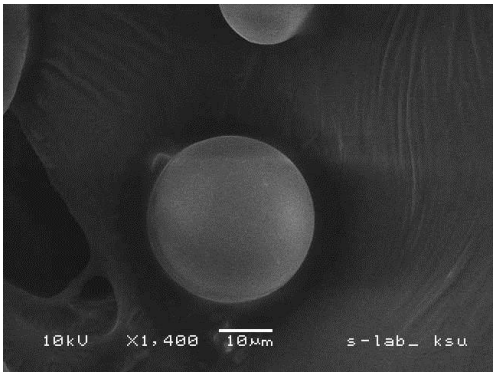


Figure 2.6. SEM images for AmB-NPs prepared by RGPd 50105 copolymer (A6); RGPt 50106 copolymer (B6); RGPd 50155 copolymer (C6) and GPd 5055 copolymer (D6) formulations

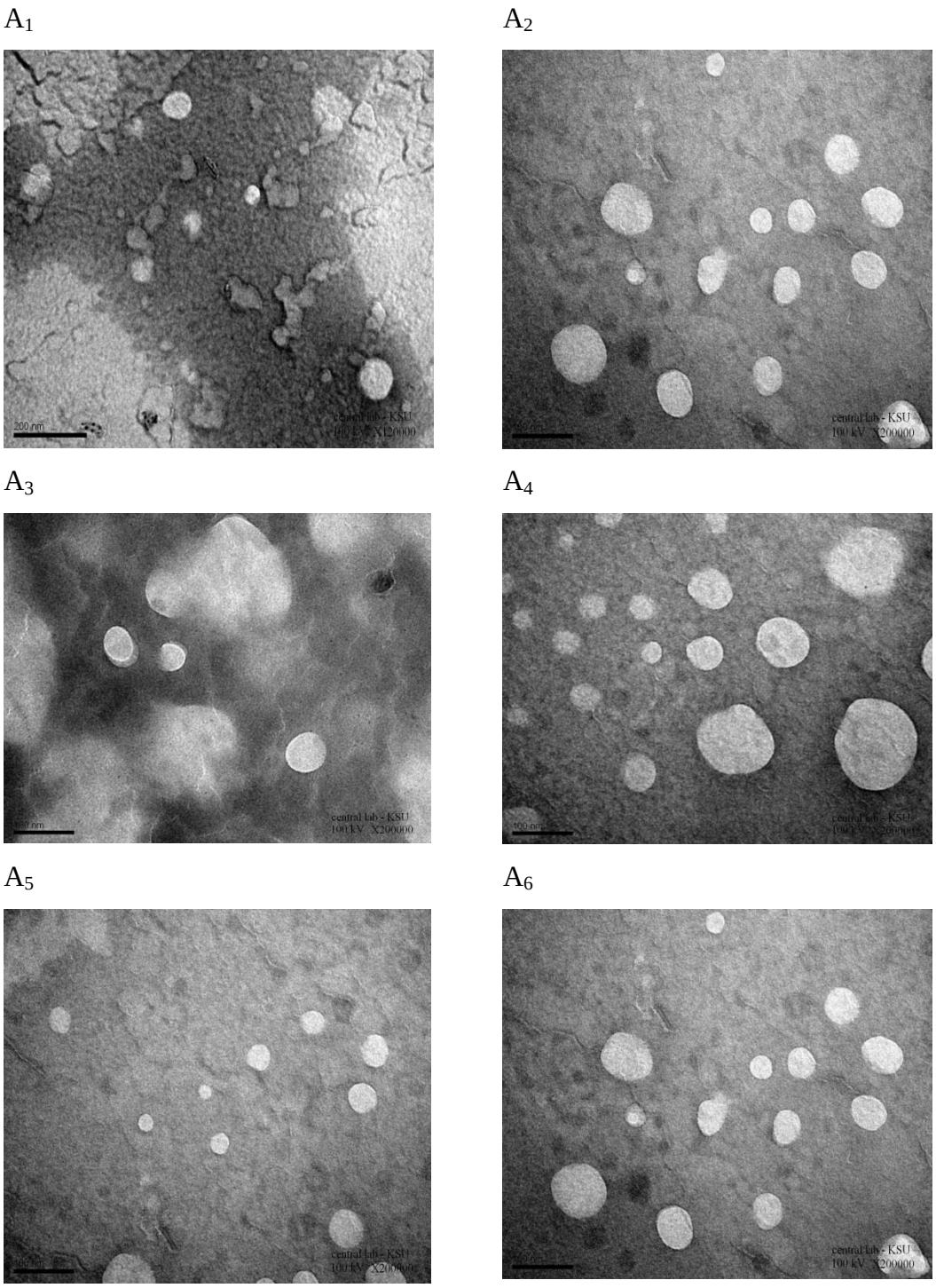
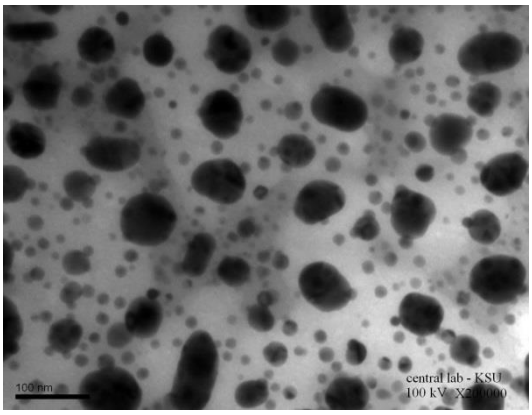
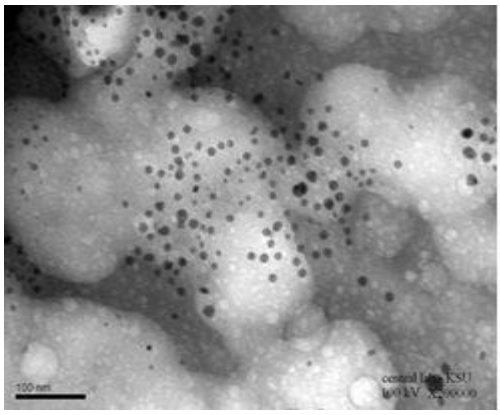


Figure 2.7. TEM images for AmB-NPs prepared by RGPd 50105 copolymer (A) formulations

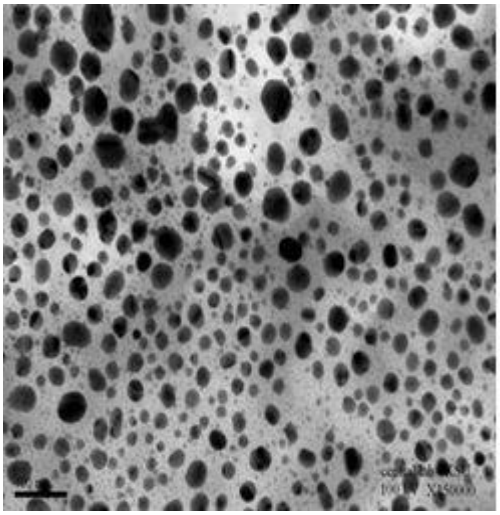
B_3



B_4



B_5



B_6

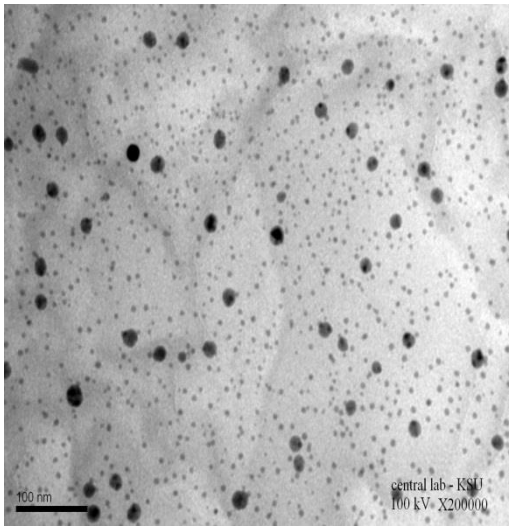
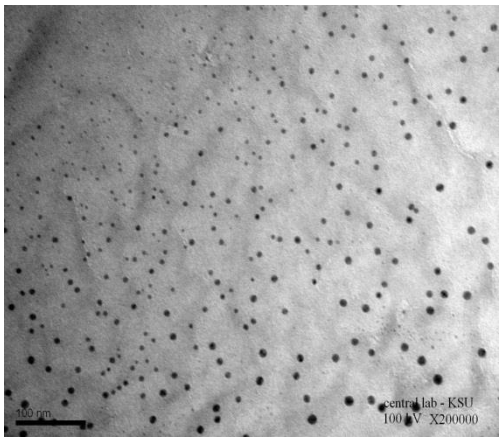
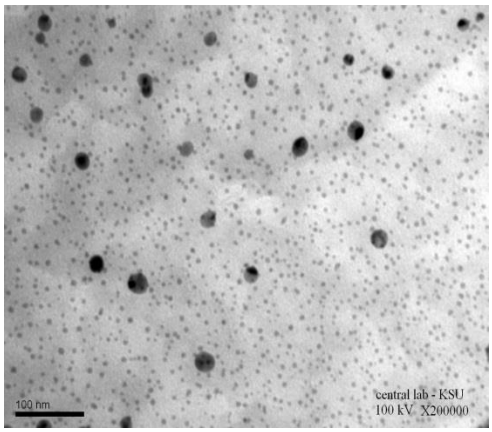


Figure 2.8. TEM images for AmB-NPs prepared by RGPt 50106 copolymer (B) formulations

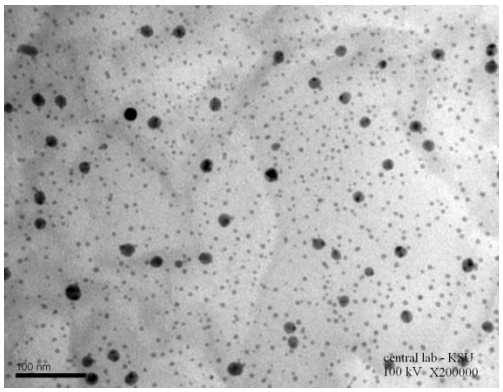
C_3



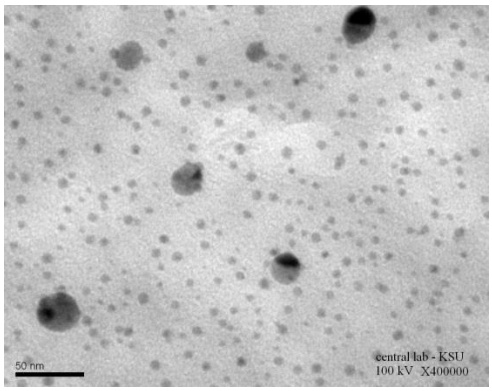
C_4



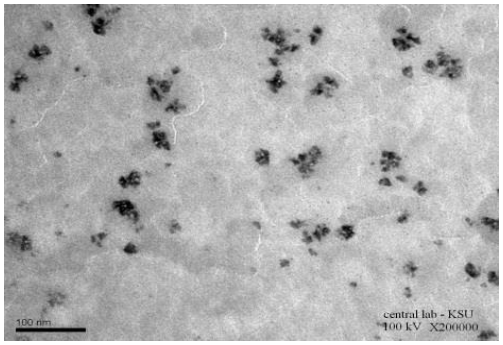
C_5



C_6



C_7



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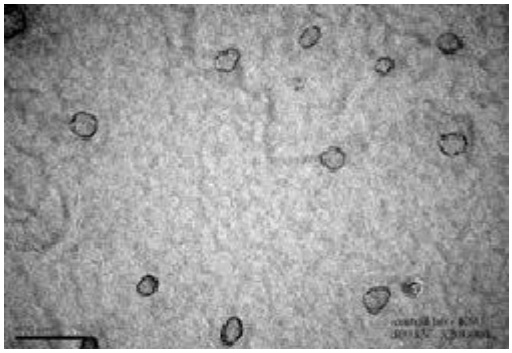
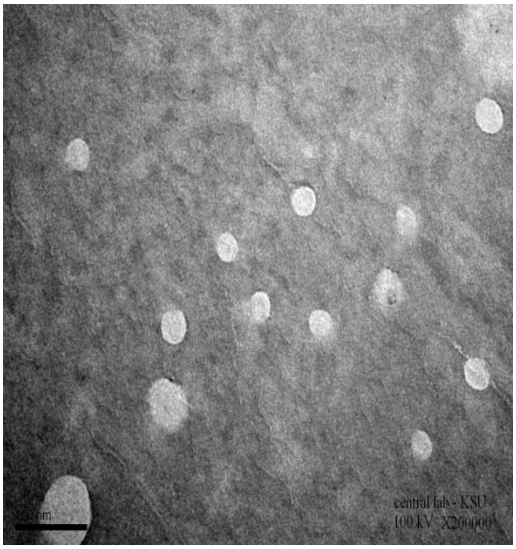
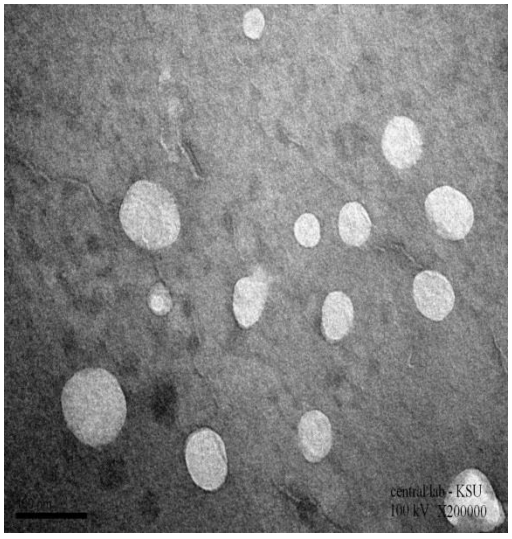


Figure 2.9. TEM images for AmB-NPs prepared by RGPd 50155 copolymer (C) formulations

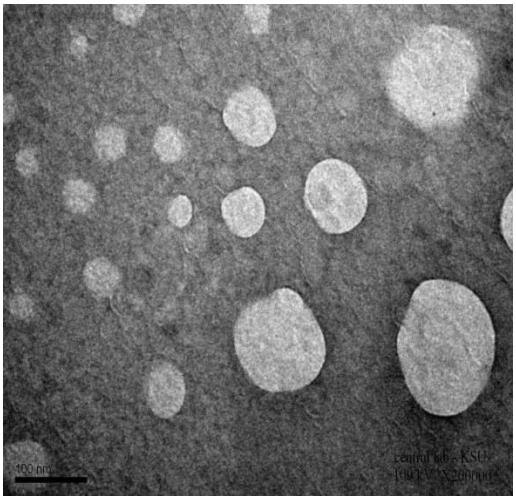
D_3



D_4



D_5



D_6

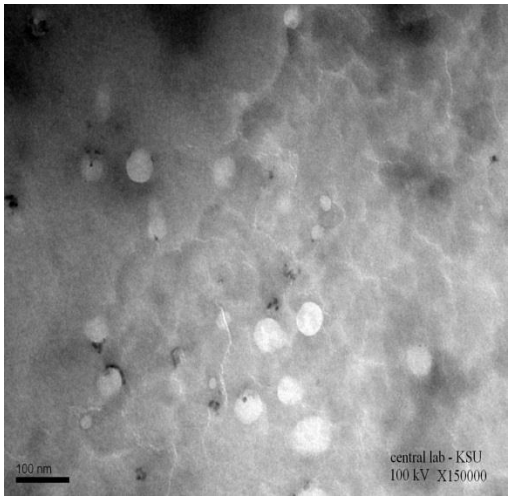


Figure 2.10. TEM images for AmB-NPs prepared by RGPd 5055 copolymer (D) formulations

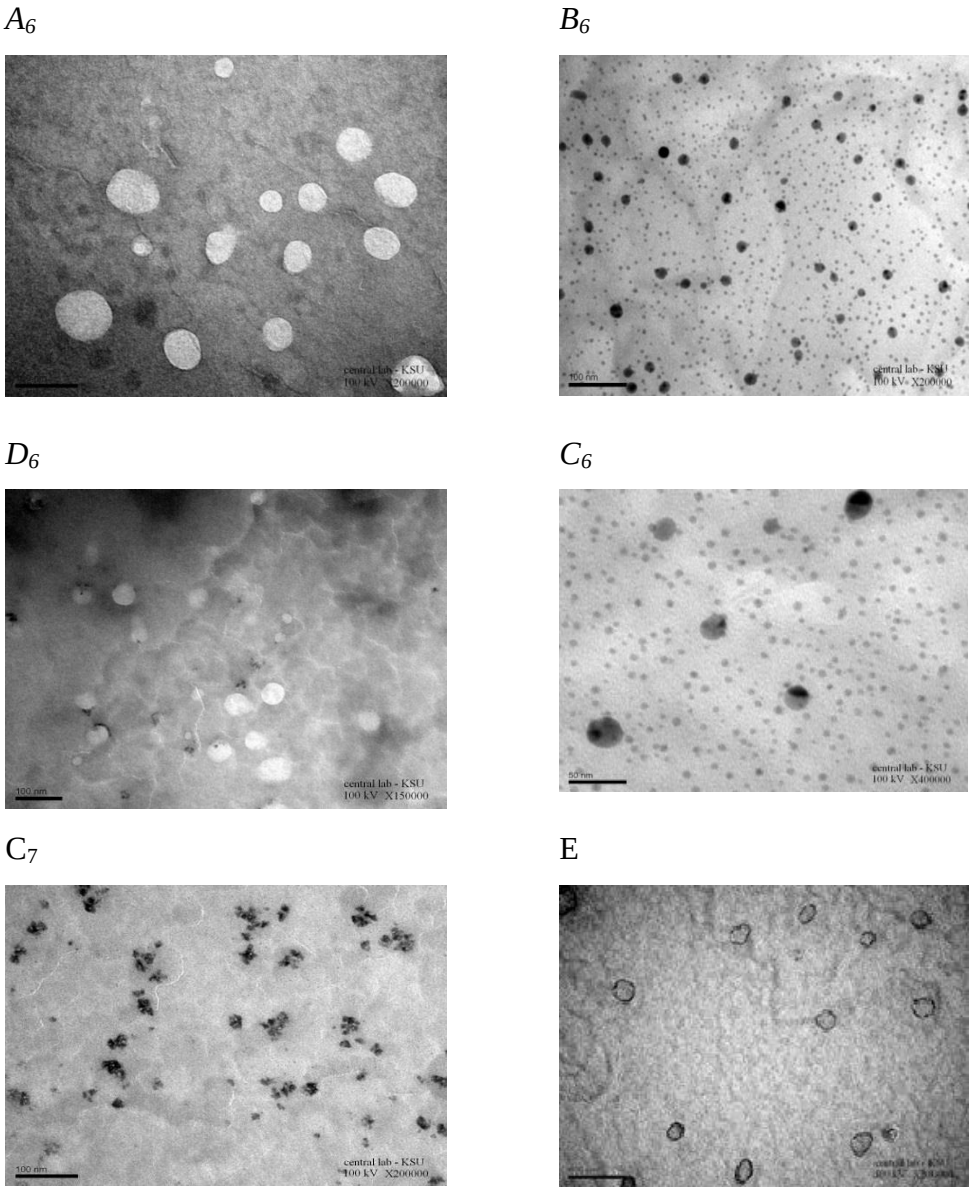


Figure 2.11. TEM images for AmB-NPs prepared by RGPd 50105 copolymer (A6); RGPt 50106 copolymer (B6); RGPd 50155 copolymer (C6) and GPd 5055 copolymer (D6) formulations

2.9.8. Fourier Transform Infra-Red (FTIR) Analysis

FTIR was used to identify AmB and to detect any change in the drug in the formulations compared to the pure drug. Therefore, the analysis was carried out for both pure AmB and pure copolymer used throughout the study, and then compared to the freeze dried formulations of AmB-NPs.

The spectra of the AmB was similar to those found in the literature (Asher and Schwartzman, 1977). The functional groups (the important bands) characteristic of AmB of the FTIR spectroscopy are shown in Figure 2.12 and listed in Table 2.10. The FTIR spectrum of AmB confirmed the presence of carboxylic, amine and ester groups.

The peak at 3500 cm^{-1} was due to the N–H stretching of the amine groups. The peak at 3390 cm^{-1} was due to the C–H stretching of the polyene and O-H stretch. The peaks at 3030, 2988 and 2824 cm^{-1} represent the C–H stretching vibrations of the alkene and alkane groups, and C–N stretching vibration appears at 1350 cm^{-1}. The peak at 1585 cm^{-1} can be assigned to the N–H stretching vibration of amine groups and C=C of alkene groups. The peaks at 1670, 1480 and 1242 cm^{-1} represent C=O, OH and C–O stretching vibrations of carboxylic groups. The peaks at 1750 and 1150 cm^{-1} represent C=O and C–O bonds, respectively, indicating the existence of an ester group. The peak at 1050 cm^{-1} was associated with the C–OH bond present in AmB (Asher and Schwartzman, 1977).

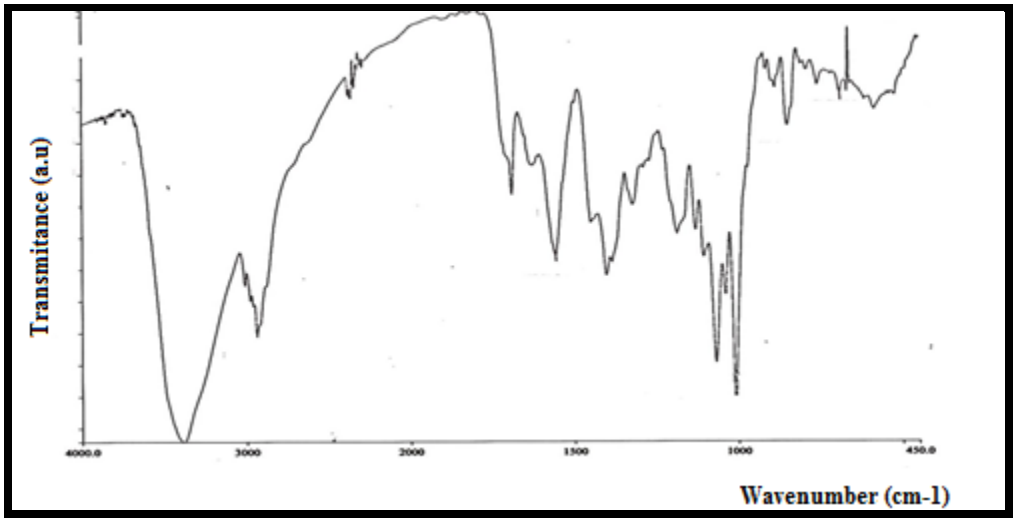


Figure 2.12.FTIR spectra of AmB alone

Table 2.10. Important bands of IR spectrum of Amphotericin B*

Frequencies (cm^{-1})	Functional group present
3500	C-N stretch of the amine group
3390	C-H stretch (polyene) and O-H stretch (strongly H-bonded)
3030	C-H stretch vibrations of the alkenes
2940	C-H_3 asymmetric shoulder stretch band
1691	Sharp C=O stretch band, NH_2 in-plane bend
1557	Polyene C=C stretch band
1402	C-H bend in polyene ring
1069	C=O asymmetric stretch
1009	C-H bend out of plane bend (trans polyene)
851	C-H bend in pyranose ring vibration

*(Nahar and Jain, 2009)

Figure 2.13 shows the FTIR of pure product of AmB and the copolymer used (PLGA-PEG copolymer). This Figure indicates that AmB has characteristic sharp bands at 1691, 1557 and 1009 cm^{-1}, responsible for the sharp C=O stretch band; NH2 in-plane bend, polyene C=C stretch band and C-H bend out of plane bend (trans polyene), respectively. The PLGA-PEG copolymer produces minor IR absorption in this region. Therefore, any change in this region could be used to detect any sort of interaction between AmB and copolymer in any formulation tested.

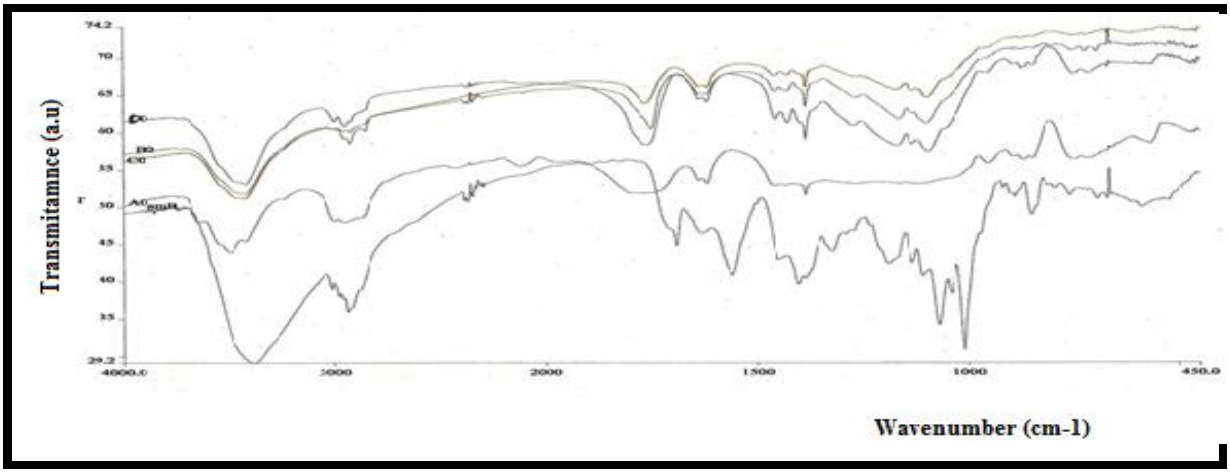


Figure 2.13. FTIR spectra of AmB and the four different type of PLGA-PEG copolymer each one alone.

Figure 2.14-2.17 display the FTIR spectra for AmB NPs prepared by RGPd 50105 copolymer (A); RGPt 50106 copolymer (B); RGPd 50155 copolymer (C) and RGPd 5055 copolymer (C). The FTIR spectrum for the pure AmB is different compared to that of AmB-NPs. The pure AmB crystals shows characteristic sharp bands at 1691, 1557 and 1009 cm-1, due to a C=O stretch band; NH2 in-plane bend, polyene C=C stretch band and C-H bend out of plane bend (trans polyene), respectively. This is in agreement with FTIR of pure AmB published by the following authors (Asher and Schwartzman, 1977, Nahar and Jain, 2009).

In case of the freeze-dried formulations of AmB-NPs, in particular, the carbonyl-stretching band was lost likely due to an interaction between the AmB and the polymer used. Similar interactions were observed with all the copolymers used.

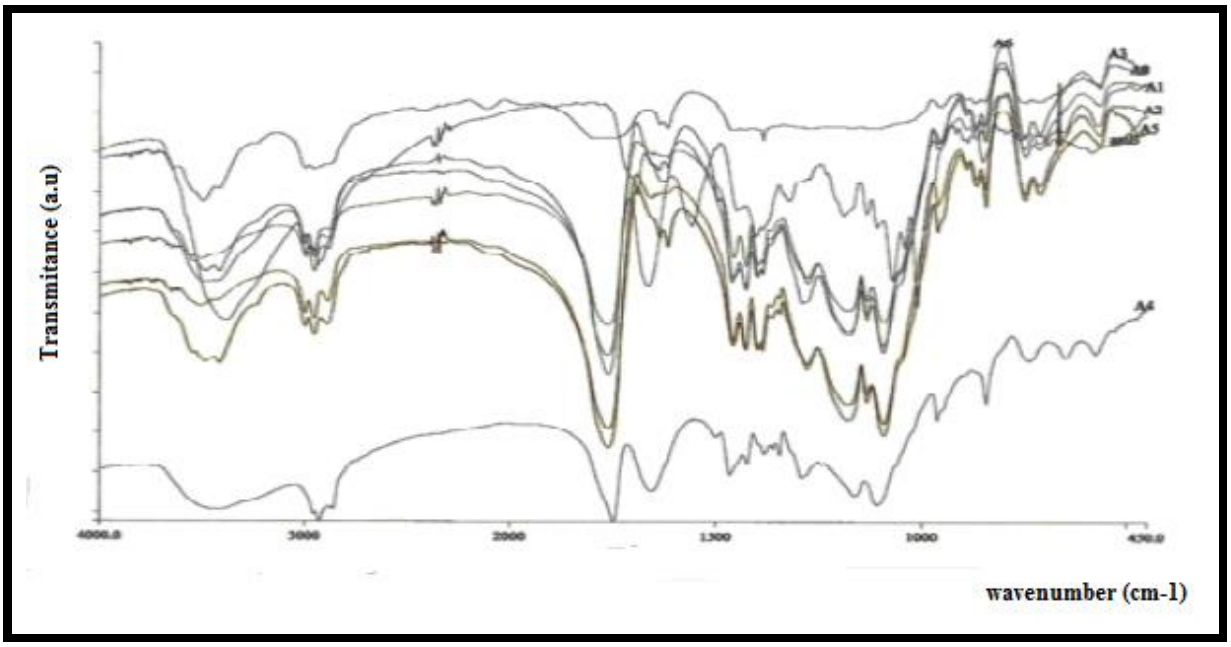


Figure 2.14.FTIR spectra for AmB-NPs prepared by RGPd 50105 copolymer (A) formulations.

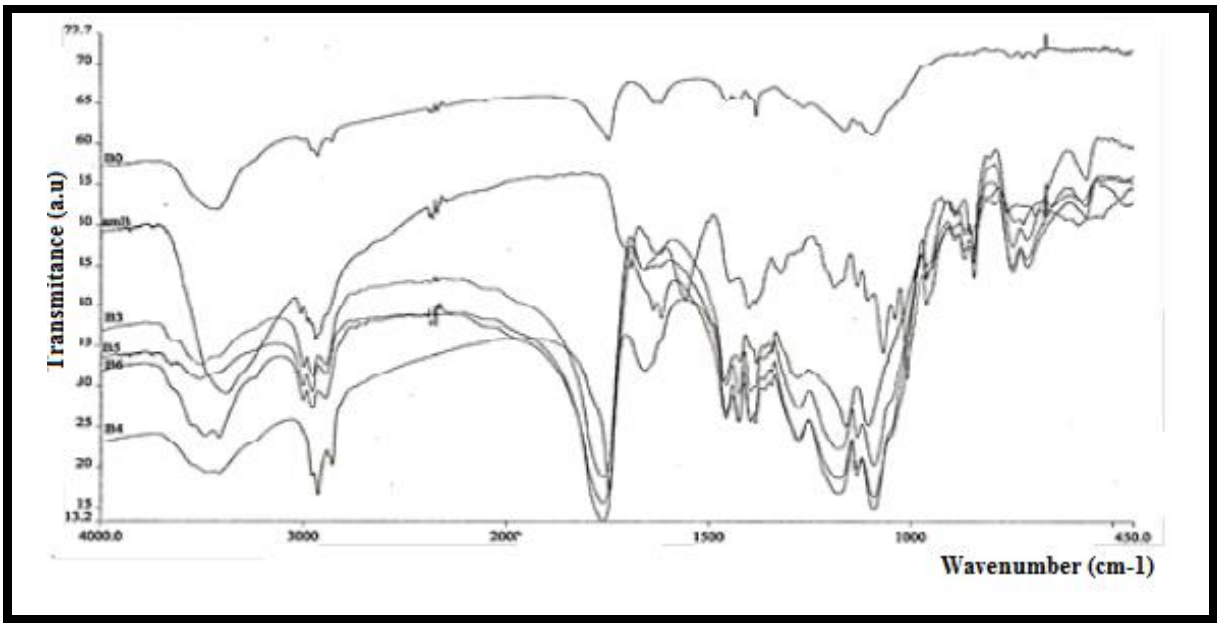


Figure 2.15. FTIR spectra for AmB-NPs prepared by RGPt 50106 copolymer (B) formulations.

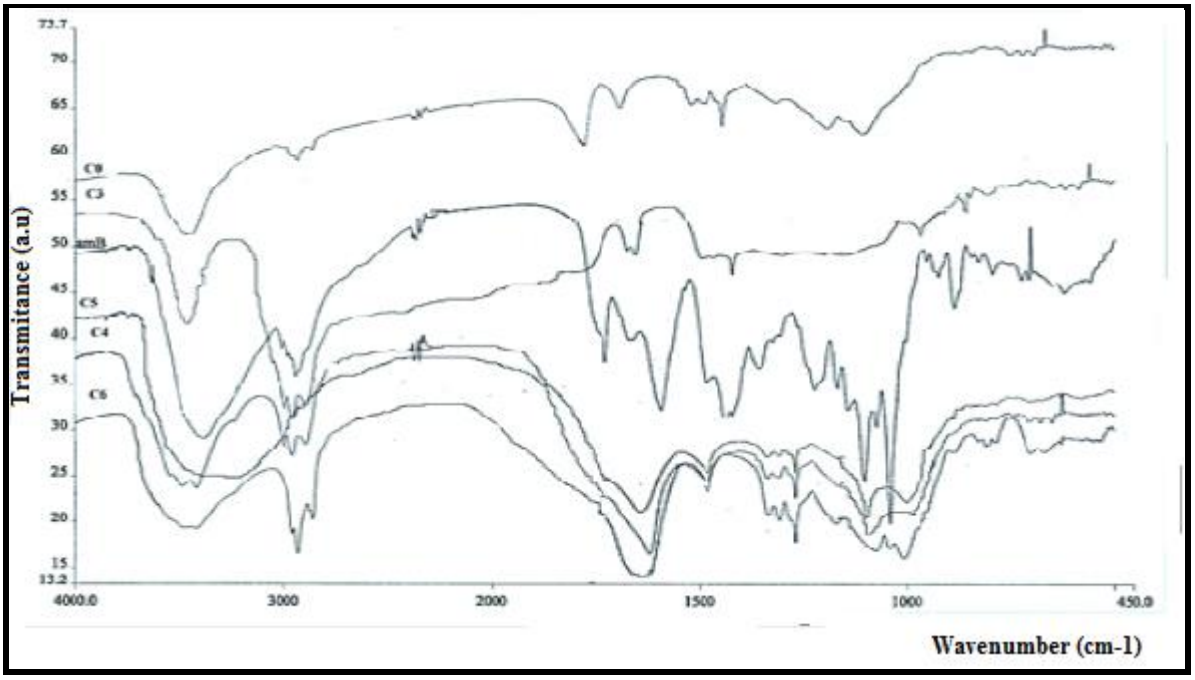


Figure 2.16.FTIR spectra for AmB-NPs prepared by RGPd 50155 copolymer (C) formulations.

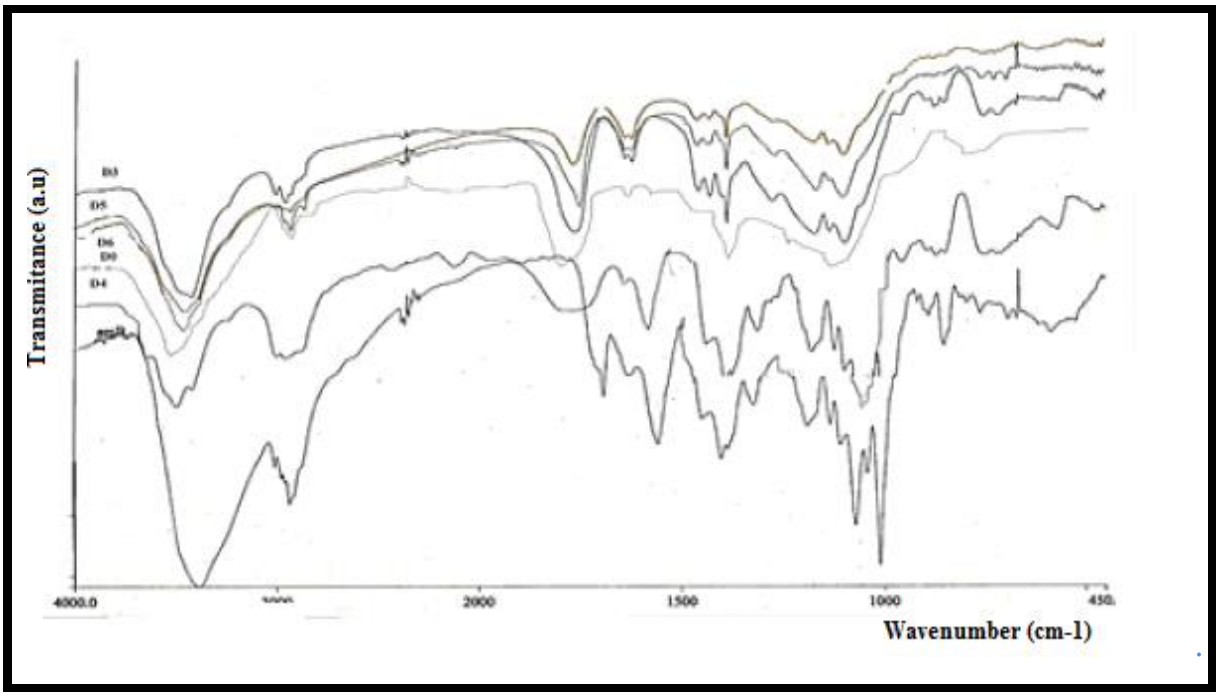


Figure 2.17.FTIRspectra for AmB-NPs prepared by RGPd 5055 copolymer (D) formulations.

2.9.9. Differential Scanning Calorimetry (DSC)

DSC is an important tool to determine the compatibility and stability of the drug with the used excipients. Figure 2.18 shows the thermogram of AmB and the copolymer, each separately. AmB is hygroscopic in nature; therefore, starting from 30°C to 100°C, AmB has a broad endothermic peak due to the loss of moisture of adsorbed water. The two characteristic endothermic peaks were observed for AmB at 168.5°C and 213.4 °C which closer to the melting point of AmB crystal as indicated in the literature(Asher and Schwartzman, 1977, Janoff et al., 1988, Madden et al., 1990, Salerno et al., 2013).

The DSC traces for the pure copolymers did not show any endothermic peak in the melting region of AmB. Meanwhile, characteristic peaks for the copolymer at 66.3 - 68.7 °C were detected, corresponding to its phase transition temperature (Tm). AmB- NPs formulations showed a shift to the right in AmB endotherm (Figure 2.19), which is indicative of shielding of AmB by the matrix constituents (Al-Assady et al., 2013). This is due to the loss of the crystalline lattice of AmB and the formation of an amorphous state as a result of the incorporation of AmB inside the copolymer. Many studies have reported that

the amorphous form enhances the dissolution and bioavailability of drugs because of higher Gibbs free energy in the amorphous form(Kim et al., 2008, Khadka et al., 2014). Patterson et al. prepared the amorphous forms of three poorly water soluble drugs (carbamazepine, dipyridamole and indomethacin) with PVP. Their dissolution rates were higher than their non amorphous form(Patterson et al., 2007).

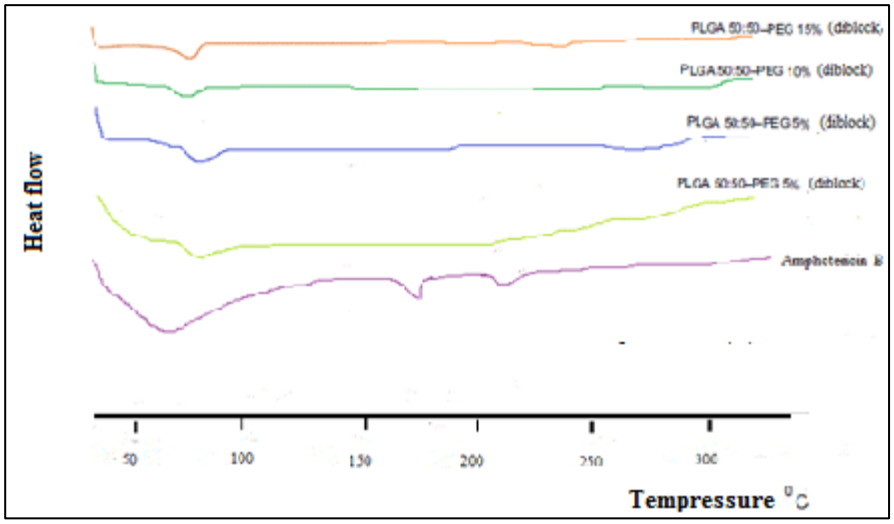


Figure 2.18. DSC thermograms of Amphotericin B and pure copolymer used.

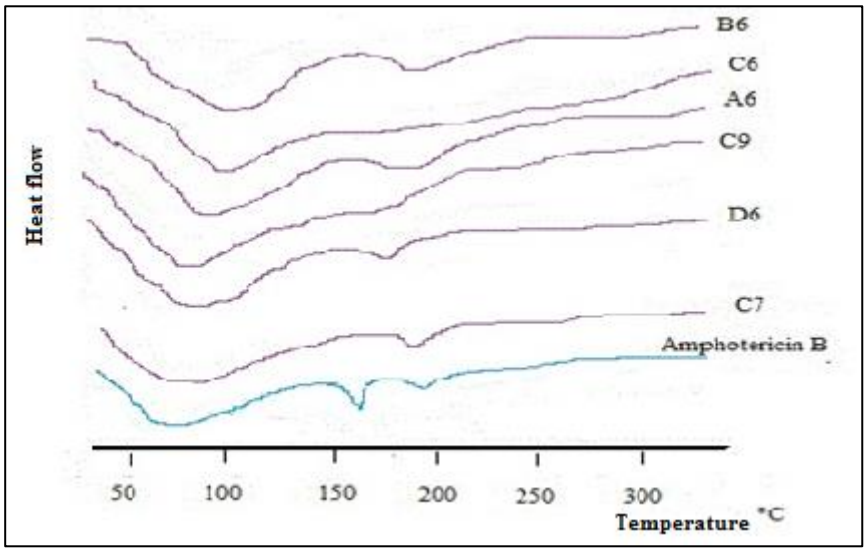


Figure 2.19.DSC thermograms of Amphotericin B and AmB-loaded NPs with RGPd 50105 copolymer (A6); RGPt 50106 copolymer (B6); RGPd 50155 copolymer (C6) and GPd 5055 copolymer (D6) formulations.

2.9.10. Chromatographic Analysis for Amphotericin B

Since AmB is only available as parenteral dosage form, only one HPLC method has been published for the determination of AmB in pharmaceutical dosage forms (Zhan-rui et al., 2009).

High-performance liquid chromatography (HPLC) developed during the 1960s and it is perhaps the most versatile analytical technique available to the modern analysts.

A new sensitive HPLC method for AmB with Nicardipine as IS was developed in this study. This method was used for the quantitative determination of AmB concentration for its *in vitro* studies as drug content analysis, percentage yield and amount of AmB released during the dissolution study.

It was noticed that decreasing the proportion of acetonitrile in the mobile phase than the amount used was resulted in peak broadening. In the meantime, increasing the proportion of acetonitrile led to early elution of AmB along with other interfering peaks. The method was found to be specific for AmB with no interfering peaks from any constituents of the NPs were co-eluted, during *In vitro* studies, with AmB or IS peaks which are a further confirmation of the specificity of the method.

Although there is no structural similarity between AmB (Lemke et al., 2005) and Nicardipine (Fernandez et al., 1990) (Figure 2.20), they have shared similarity in the solubility behavior in the mobile phase used and detected at the same wavelength, which makes Nicardipine is an appropriate choice as the IS for this study.

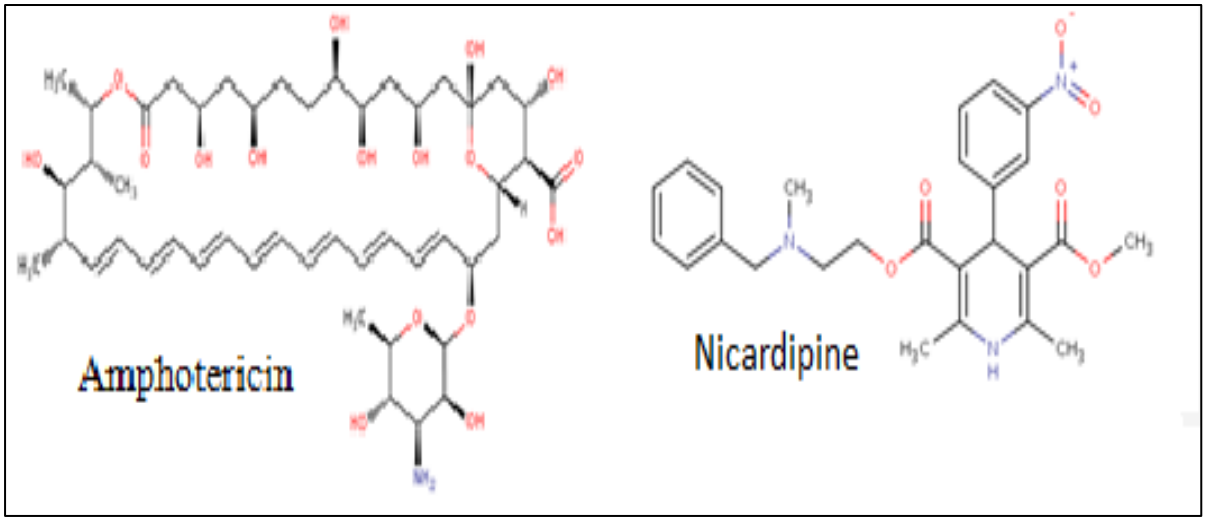


Figure 2.20. Chemical structure of amphotericin B (Lemke et al., 2005) and Nicardipine as IS(Fernandez et al., 1990).

It is known that maximum UV absorbance for AmB at 383 and 407 nm. The HPLC analytical methods reported in the literature often detect AmB either at 407nm or 383 nm. In this study, AmB was better detected, with better sensitivity, at 382nm wavelength. Therefore, 382 nm was selected to identify AmB in the HPLC assay.

Figure 2.21, shows the chromatogram A, for the blank, mobile phase, and chromatogram B represents AmB and IS with the average retention times of 2.87 ± 0.05 and 4.20 ± 0.09 min, respectively with no interfering peaks. This is an indication the specificity of the HPLC assay method. Others published HPLC methods for AmB determinations need longer time for detection of AmB alone, with exception of Balbathula et al., (Balabathula et al., 2013)where the retention time is 2.7 minutes but the method required careful sample preparation in order to reduce the chances of errors during analysis.

chromatogram A chromatogram B

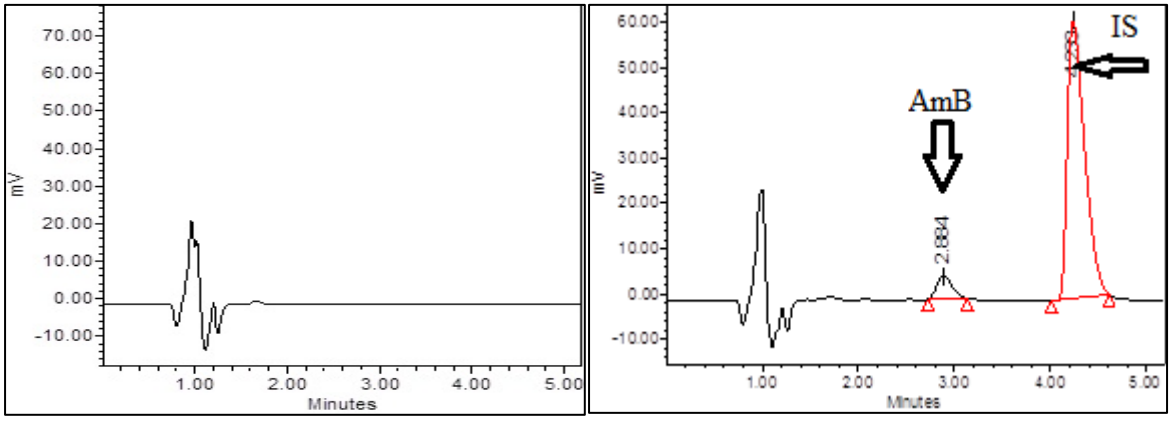


Figure 2.21. HPLC chromatograms of mobile phase (chromatogram A) and HPLC chromatograms of mobile phase containing 0.5 µg/ml amphotericin B and 10 µg/ml Nicardipine as internal standard (chromatogram B).

2.9.10.1. HPLC Methodology Validation

A calibration curve of peak area ratios, of AmB to IS, versus concentrations, ranging from 0.25 to 5.0 µg/ml, was plotted in Figure 2.22. Each point on the calibration curve represents a mean of six determinations. A linear curve was obtained for the range of concentration tested with a correlation coefficient (*r*) of 0.999.. The regression equation was Y = 0.379X – 0.04.Therefore, good linear relationships between the AmB peak area ratios and its tested concentrations.

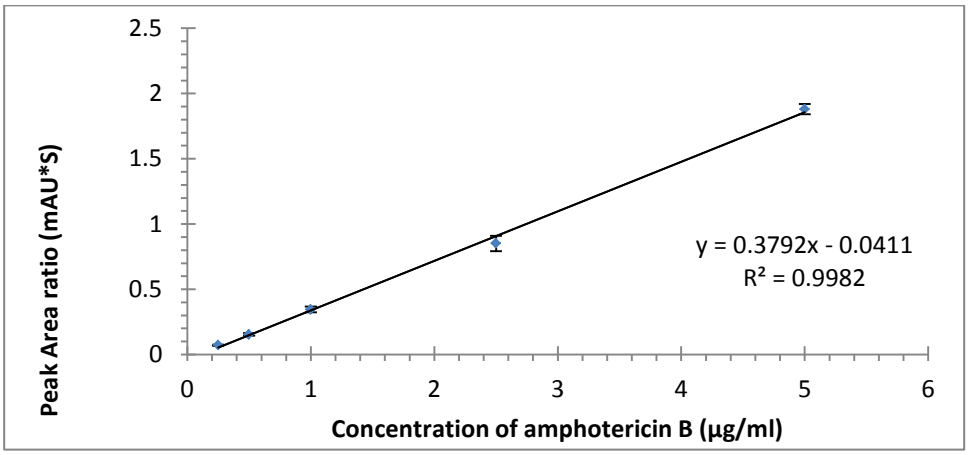


Figure 2.22. Standard calibration curve of *in vitro* AmB in methanol at λ 382 nm (n = 6)

2.9.10.2. Precision and Accuracy

The closeness or similarity in a series of measurements derived from multiple sampling of the same homogenous solution under similar conditions is an indication of an assay precision. The interday and intraday precision are measures of method variability that can be expected when it is used for analysis (Shaikh et al., 2008) and is expressed by the RSD. %. The intraday precision (Table 2.11) was determined by analyzing six replicates each of 0.25, 1.0 and 5 µg/ml concentrations of AmB solution. For interday precision determination (Table 2.12), six replicates each of concentrations of AmB were analyzed on three different days. The relative standard deviations were determined. For the determination of accuracy, the relative error was calculated (Health and Services, 2001). The relative standard deviation RSD% values obtained range from 4.4 – 6.2 for intraday precision and 4.2 – 5.8 for interday precision respectively and indicate good precision for the developed method.

Table 2.11. Intraday precision determination (n = 6)

Concentration (µg/ml)	0.25	1	5
Average	0.071	0.350	1.825
SD	0.004	0.015	0.114
RSD%	5.193	4.436	6.278

Table 2.12. Interday precision determination (n = 6)

Concentration (µg/ml)	0.25	1	5
Average	0.071	0.353	1.82
SD	0.003	0.015	0.105
RSD%	4.225	4.323	5.789

The accuracy, calculated as the relative error of the average concentrations determined by HPLC compared to standard solutions of AmB was determined at low, medium and high concentrations (i.e. 0.25, 1.0 and 5.0µ g/ml, respectively) of AmB solution. The relative standard deviation RSD % values ranging from 3.3 to 6.1for intraday accuracy (Table 2.13) and 2.7 to 5.66 for interday accuracy (Table 2.14) indicate that the method is sufficiently accurate for determination of AmB in solutions.

Table 2.13. Intraday accuracy determination (n = 6)

Concentration (µg/ml)	0.25	1	5
Average	0.29	1.03	4.92
SD	0.009	0.04	0.30
RSD%	3.29	3.97	6.14

Table 2.14. Interday accuracy determination (n = 6)

Concentration (µg/ml)	0.25	1	5
Average	0.29	1.04	4.91
SD	0.008	0.04	0.28
RSD%	2.68	3.87	5.66

2.9.10.3. Robustness

The assay method was robust, since a small-intended change in assay conditions as changing the column or the HPLC system has not shown any significant effect on the chromatographic performance of AmB. Even a little difference in the mobile phase composition did not show any noticeable effect in peak area ratio of AmB.

2.9.11. In vitro Release of Amphotericin

In vitro drug release profiles an imperative test to predict the *in vivo* performance of a drug delivery system. An *in vitro* drug release study is indeed a prerequisite for a drug design optimal effect (Kumari et al., 2010). The drug release pattern was studied for 24 hours for all formulations.

AmB is not available as oral dosage form in the market, therefore, there is no available official dissolution medium for it. Several authors have been investigated different dissolution media for AmB *in vitro* release studies. Simulated intestinal fluid with pancreatic enzymes (SIFe) (pH = 7.5) was used as the dissolution medium (Tiyaboonchai et al., 2001, Zu et al., 2014). Furthermore, dissolution medium can be 500 ml of 0.1 N HCl (Singh et al., 2013). For the lipid-based formulations, the simple dissolution assessment media would not be appropriate to evaluate the absorption of these preparations. Ibrahim and his group have indicated that the presence of phospholipids, bile salts and lipase enzyme in dissolution media maintained at 37 ^{0}C and at constant pH are essential for their

dissolution(Ibrahim et al., 2012).Other researches, used equilibrium dialysis membrane for AmB dissolution studies (Nahar et al., 2008). The release medium used in this work is consisted of 2% sodium deoxycholate (SDC) in phosphate buffer (0.1 M, pH 7.4) which is similar to the one reported by Jain and Kumar(Jain and Kumar, 2010).

The *in vitro* release profiles of AmB in phosphate buffer with 2% sodium deoxycholate are depicted in Figure 2.23- 2.26. The differences observed in release profiles may be due to differences in the type of polymer used, percentage of PEG in the polymer, and presence of different additives in the formulations.

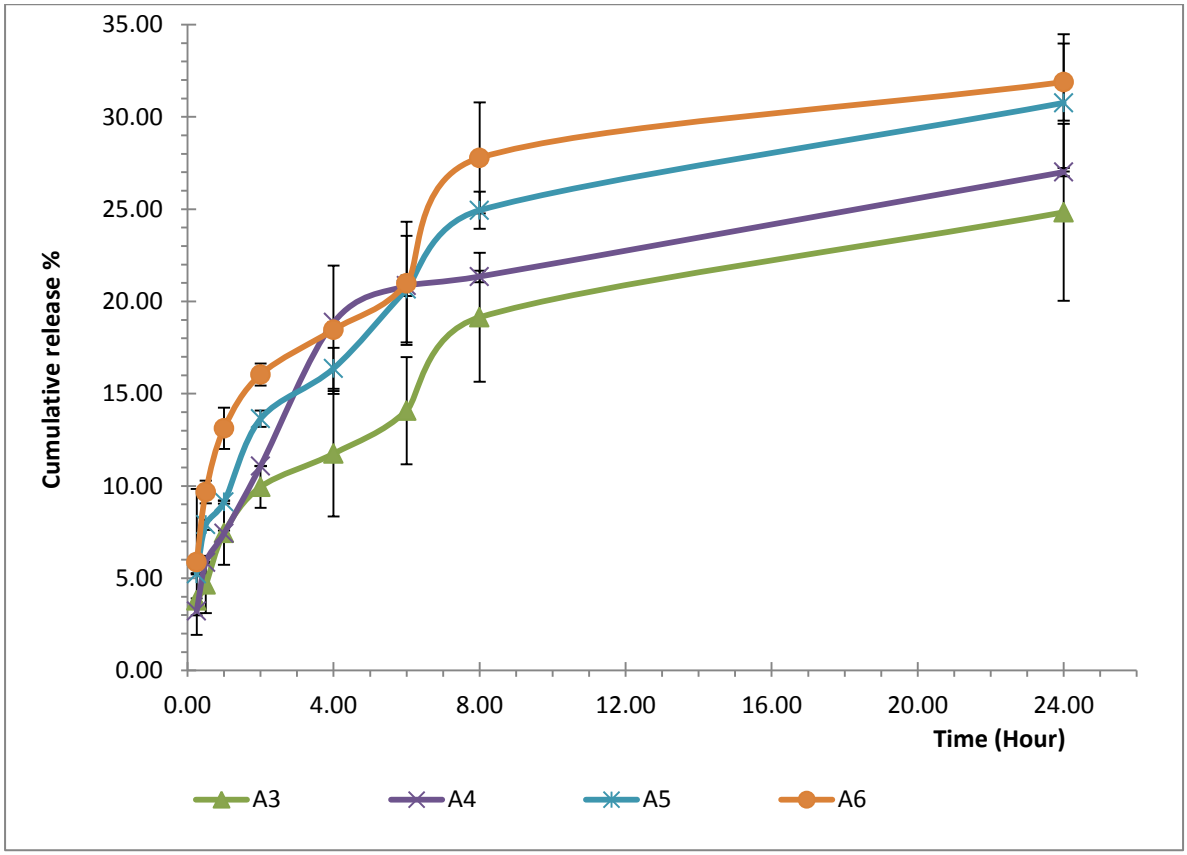


Figure 2.23. *In vitro* drug release behavior for AmB-NPs prepared by RGPd 50105 copolymer(A) in phosphate buffer with sodium deoxycholate (2%).

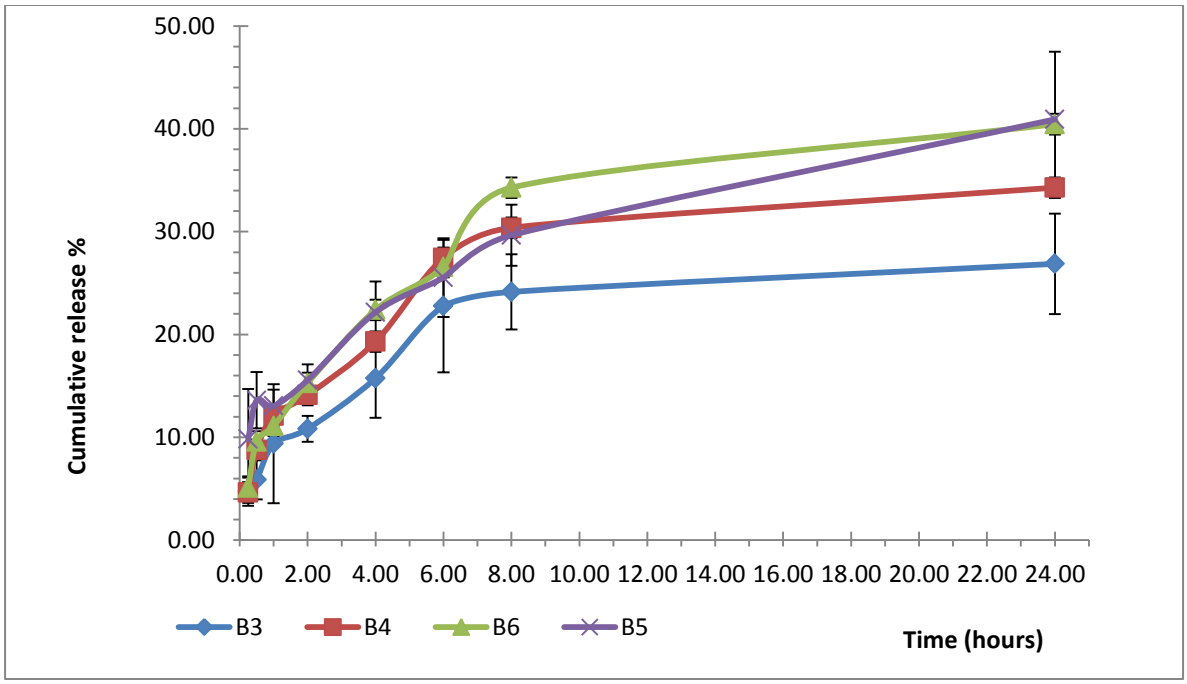


Figure 2.24. *In vitro* drug release behavior for AmB-NPs prepared by RGPt 50106 copolymer (B) in phosphate buffer with sodium deoxycholate (2%).

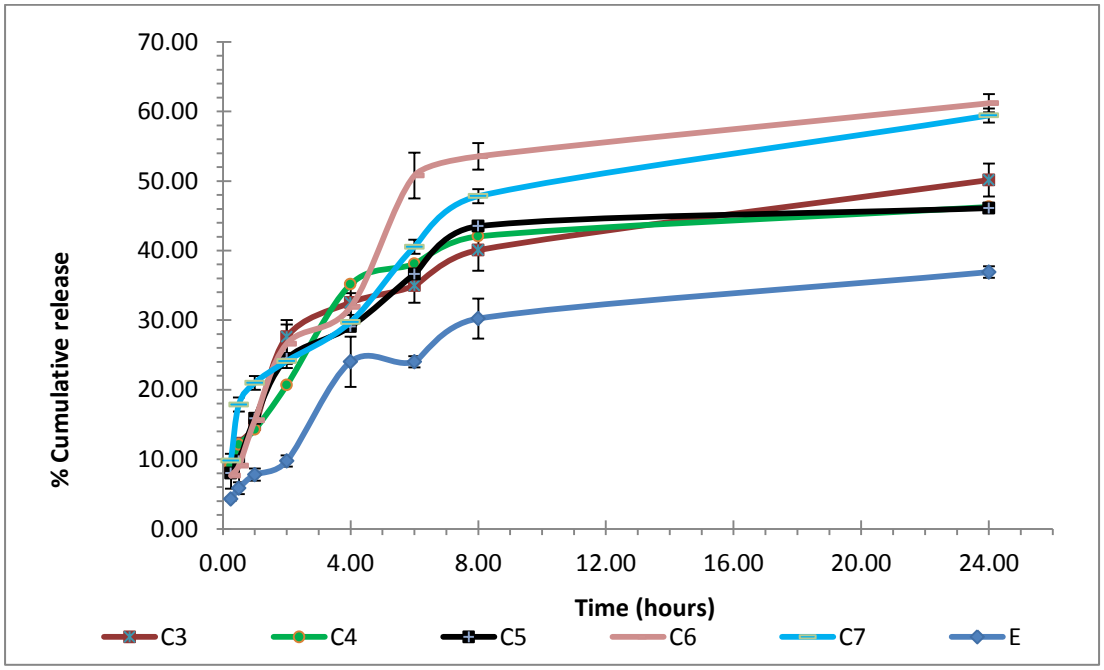


Figure 2.25.*In vitro* drug release behavior for AmB-NPs prepared by RGPd 50155 copolymer (C) and R 203 H (E) in phosphate buffer with sodium deoxycholate (2%).

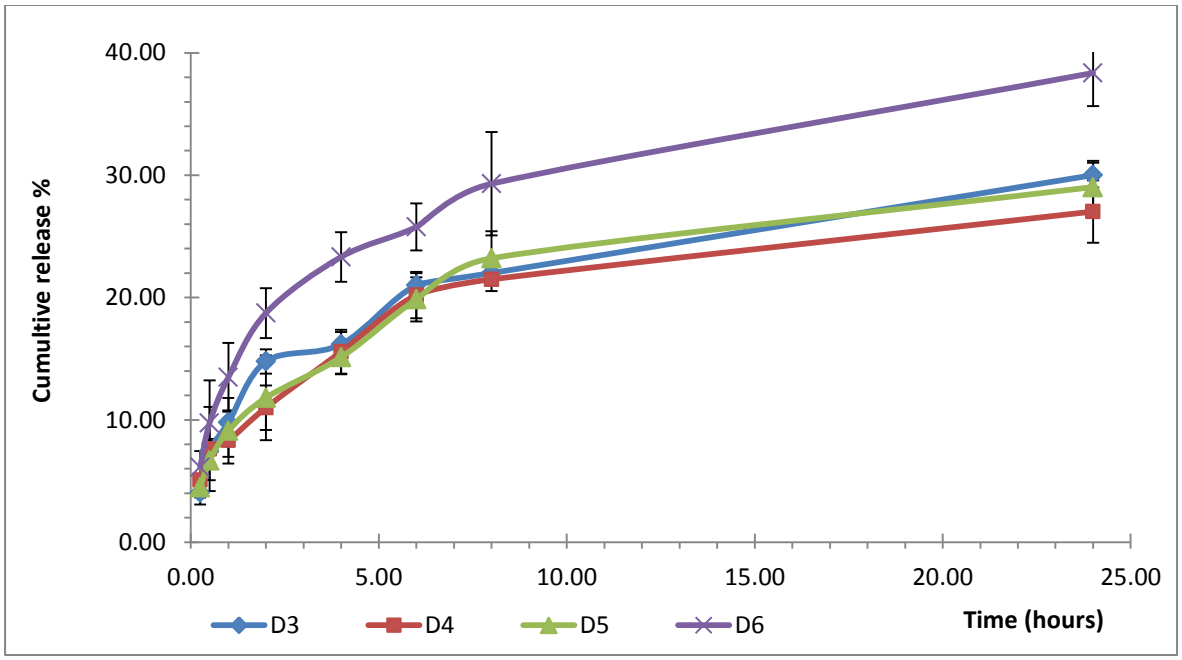


Figure 2.26.*In vitro* drug release behavior for AmB-NPs prepared by GPd 5055 copolymer (D) in phosphate buffer with sodium deoxycholate (2%).

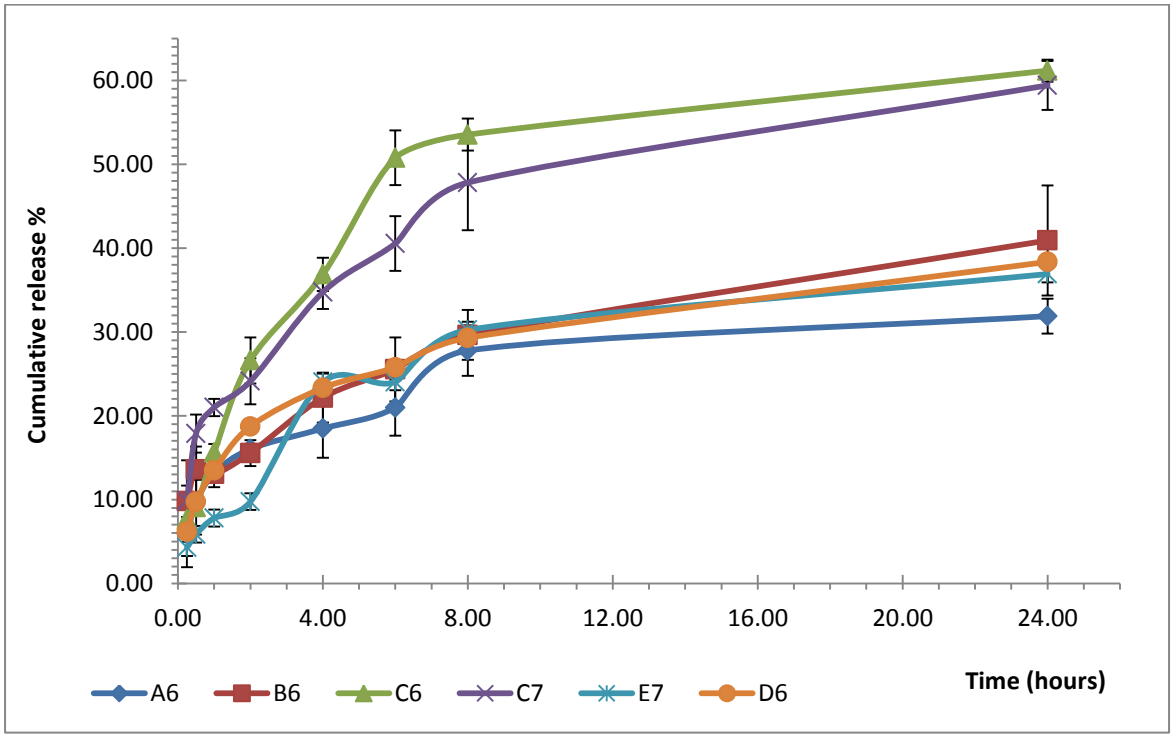


Figure 2.27.*In vitro* drug release behavior for AmB-NPs prepared by selected copolymer in phosphate buffer with sodium deoxycholate (2%).

The release profiles of AmB from NPs formulations indicate biphasic release. Where, in the first phase (6 h, R^2>0.9) there was an initial rapid release of about 21-51%, depending on the formulation, followed by a slower phase, from 6-24 h, where only about 11% was released. No lag time was observed (4-10% release in the first 15 min) which could be attributed to the existence of some of the AmB adsorbed on the surface of the NPs or the ability of the dissolution medium, containing the surfactant, to aid the initial release of the drug from the polymer. It should be mentioned that, removal of the surfactant from the dissolution medium resulting in minimum release AmB (< 7 %). The highest drug release within 24 h was obtained with C6 (61%), while the lowest one was A3 (24.8%).

Regarding the release of AmB loaded to PLGA-PEG copolymer (A) formulations as shown in Figure 2.23, a significant (P<0.05) increment in the order of A3 (24.8%) < A4 (27%)< A5 (30.7%)< A6 (31.9%) was obtained. The same pattern was observed with other PLGA-PEG copolymer used (B, C and D). The existence of this unique pattern because of the different stabilizers added to the AmB-NPs formulations.

Comparing the six different formulations of the four PLGA-PEG copolymer, formulations number 3 (A3, B3, D3 and C3) showed the lowest AmB release among them A3 (24.8%) < B3 (26.9%) < D3 (30%) < C3 (50%). While the highest AmB release among them C6 (61.2%) > B6 (40.9%) < D6 (38%) > A6 (31.9%).

The addition of miglyol-812 (2.5%) to the basic component of each copolymer (Formulations A4, B4, C4, D4) during the preparation process has led to a significant (P<0.05) increase in AmB release. The same trend was observed on adding TPGS (5% w/v) causing further increment in release behavior of A4 (27%) < D4 (27%) < B4 (40.4%) < C4 (46.3%). Moreover, increasing the concentration of PVP from 1% to 4%, the drug release of AmB from the NPs formulations was also increased < D5 (29%) <A5 (30.7%) <, B5 (40.9%) < C5 (46.1%) significantly (P<0.05). A similar trend was reported for several hydrophobic drugs after the addition of miglyol-812, such as ketoprofen (Patil et al., 2004), simvastatin(Zhang et al., 2010b) and lacidipine (Basalious et al., 2010).The same observation, of noticeable improvement, in the *in vitro* release of drugs was recorded upon the addition of TPGS to different formulations (Mu and Feng, 2002, Mu and Feng, 2003, Zhang et al., 2008, Tang et al., 2014).

Increasing the PVP content from 1 to 4% in AmB loaded to PLGA-PEG increased the release rate (32%) C6 > (32%) D6> (28.8%) C7 > (3.67%) A6 > (1.15%) B6. This finding is in agreement with the results of different researchers group for drug such as terbinafine and protein (Arunprasad et al., 2010, Shakeri et al., 2015).

The more the reduction in the MPS, the larger surface area in contact with the dissolution medium, the higher the drug release will be. There was doubling AmB content in the NPs from 20 mg to 40 mg in formulations C7. showed non-significant (P= 0.887) difference in the AmB release rate between C6, with 20 mg AmB, and C7, with the 40 mg AmB, formulations. This means that the dissolution media could not be able to dissolve more than the amount of AmB released from C6 i.e. the maximum solubility in this dissolution medium could be reached. It should be mentioned that the increase of AmB content in the NPs shows insignificant (P=0.654) reduction of MPS between C6 and C7 which could be another explanation of the no change in the release rate as a result of increasing the drug content.

It is interestingly notice that changing the PLGA-PEG copolymer to R 203 H polymer (non-peglyated) formulation E caused a significant (P< 0.05) reduction in release rate by 95 % as compared to peglyated polymer.

Therefore, formulations A6, B6, C6, C7, E and D6 with the lowest MPS and the highest release rate were selected for further *in vivo* characterizations.

2.10. Drug Release Kinetics

To fit the release pattern of AmB-NPs different kinetic models were tested. The coefficient of determination (R^2) and release rate constant (K) were analyzed using different established models to find out the release mechanism

Table 2.15, represent the results of modeling of release profile, R^2 and K are correlation coefficient and release rate constant for particular formulation. As per expectation, AmB loaded to NPs tested formulations were found to have maximum R^2 in case of Korsmeyer–Peppas (n = 0.5) as well as Higuchi model for 24 h profile. This can be explained and the formulated AmB- NPs were considered as a matrix type system and drug

is anticipated to release by diffusion mechanism. Similar finding were by Jain and Kumar, 2010 (Jain and Kumar, 2010), when AmB loaded to (PEG)3-PLAco-polymers.

The drug release depends upon: adsorption through the nanoparticles matrix, diffusion through the nanoparticles matrix, particles erosion, a combined erosion and diffusion process and polymer degradation (chemical or enzymatic hydrolysis). The application of the correct mathematical model allows us to analyze about release rate, points of dissolution change and mechanisms of drug release.

If the diffusion process is Fickian, n is equal to 0.5, 0.45 and 0.43 for thin films, cylindrical and spherical matrices, respectively. When n exceeds these thresholds, the release is non-Fickian (Ritger, Peppas, 1987). Korsmeyer Peppas constant 'n' = 0.245 which is the beyond the limits of Korsmeyer Peppas model. It cannot be predicted clearly. It may be due complex mechanism of swelling, diffusion and erosion.

Table 2.15. Modeling of amphotericin release from different formulations in 24 hour study

Formulation code	Zero- Order		First- Order		Higuchi		Korsmeyer-Peppas		
	R^2	K	R^2	K	R^2	K	R^2	K	n
A1	-0.1863	1.538	0.0395	0.020	0.7824	6.909	0.8816	9.649	0.358
A2	-0.4214	1.203	-0.2271	0.015	0.7861	5.433	0.9479	7.993	0.334
A3	0.1271	1.307	0.2817	0.016	0.9268	5.694	0.9740	7.275	0.396
A4	-0.1558	1.510	0.0613	0.020	0.8232	6.784	0.9209	9.411	0.361
A5	-0.1974	1.676	0.0651	0.023	0.8599	7.459	0.9704	10.442	0.356
A6	-0.7106	1.777	-0.3564	0.025	0.7121	8.059	0.9532	12.493	0.311
B3	-0.3081	1.538	-0.0615	0.020	0.7751	6.959	0.9033	9.990	0.346
B4	-0.2749	1.941	0.0538	0.028	0.7967	8.750	0.9199	12.480	0.348
B5	0.0139	2.211	0.3448	0.034	0.8860	9.772	0.9501	12.895	0.382
B6	-0.4388	2.172	-0.0314	0.033	0.8120	9.634	0.9808	14.197	0.333
C3	-0.4744	2.785	0.1308	0.054	0.7705	12.604	0.9487	18.783	0.329
C4	-0.5561	2.696	0.0627	0.053	0.7019	12.365	0.8922	18.703	0.323
C5	-0.4713	2.671	0.1123	0.051	0.7341	12.200	0.9049	18.172	0.329
C6	0.0243	3.448	0.6637	0.089	0.8373	15.362	0.8943	20.120	0.386
C7	-0.2879	3.229	0.4004	0.071	0.8329	14.378	0.9642	0.9642	0.346
E	0.2356	2.007	0.4714	0.029	0.8872	8.767	0.9108	10.578	0.421

D3	-0.2474	1.622	0.0041	0.022	0.8471	7.235	0.9727	10.293	0.349
D4	-0.3310	1.491	-0.0866	0.019	0.8234	6.680	0.9630	9.624	0.344
D5	-0.0910	1.576	0.1378	0.021	0.8829	6.984	0.9704	9.515	0.368
D6	-0.4197	2.094	-0.0324	0.032	0.8111	9.410	0.9786	13.861	0.334

R^2, is the coefficient of determination; K, is the release rate constant for respective model. Where, Q_t is the amount of drug released at time t, Q_∞ is the initial amount of drug, and k is release rate constants of respective equation.

2.11. Conclusion

The first challenge in developing an oral drug delivery system of AmB was to cope with its poor solubility in any solvents. This has been overcome by co-solvation and through lowering of the pH(Venier-Julienne and Benoit, 1996, Nahar and Jain, 2009). Several other approaches have been utilized in this study to solubilize AmB loaded to PLGA-PEG copolymer including particle size reduction, addition of different absorption enhancer, the type of polymer used and the technique of preparation. The use of co-solvents is a highly effective technique to enhance the solubility of poorly soluble drugs (Millard et al., 2002, Vemula et al., 2010). The addition of a polar solvent like acetone to the dispersed organic phase decreases mean nanoparticle size and narrows the size distribution, as reported with (Venier-Julienne and Benoit, 1996). An organic mixture of both solvent ACN/DCM have been used successfully in solubilizing lipophilic drug (Hosotsubo et al., 1988).

The second challenge was to improve the drug loading efficacy (DEE) which determines the effectiveness of the system to hold the drug as well as overall amount of polymer in the carrier system required to deliver a particular amount of drug. The optimum formulation in this study has a DEE of 56.5% which is > 5 fold better compared to the liposomal AmB (AmBisome) currently used clinically, with a DEE of 11.72%(Jain and Kumar, 2010). Therefore, a novel oral AmB-NPs has been developed using a modified emulsification diffusion method using peglyated (PLGA-PEG15% -diblock copolymer) which is characterized by a simple preparation procedure, high encapsulation efficiency and high reproducibility.

Incorporation of AmB-NPs loaded to PLGA-PEG for oral delivery, displayed reduction in MPS to nanometer scale. This is in agreement with Kumar et al., (Kumar et al., 2015)study.

AmB formulation with PLGA-PEG diblock copolymer, formulation C especially which contain RGPd 50155 with 15% PEG copolymer, produced a significant ($P < 0.05$) reduction in the MPS(> 70%) compared to the other developed AmB-NPs. This could be attributed to the higher percentages of the PEG loaded in the copolymer. Similar trend was

observed by Busske and his co-authors (Buske et al., 2012). In addition, the same polymer has shown the highest drug content in comparison with other developed formulations. This is in agreement with (Schwach et al., 2004, Buske et al., 2012)where, the percentage of PEG had a significant effect on the size reduction of the formulated NPs. Meanwhile, in comparing diblock versus triblock with the same percentage of the PEG (10%), this study has shown that triblock copolymer was better in dissolution rate by about 30% than the diblock copolymer. This is in agreement with Buske and ZU groups (Buske et al., 2012, Zu et al., 2014). Therefore, PLGA-PEG 15% diblock copolymer was the best optimal formulations polymer in this study for the preparation of the AmB- loaded NPs.

The addition of miglyol to the organic phase could lead to further reduction in particle size due to the fact that miglyol®-812 (caprylic/capric triglyceride mixture) increases emulsification associated with the production of finely dispersed emulsions (Pouton, 1985). Additionally, solvent capacity for less hydrophobic drugs can be improved by blending with triglycerides, so miglyol could dissolve higher amounts of AmB. This conclusion is in agreement with the finding of (Patil et al., 2004) with ketoprofen and triglyceride tripalmitin (Garcia-Fuentes et al., 2005).The addition of a surfactant (TPGS) reduce the MPS significantly and enhanced the release of the AmB-NPs. It is reported that TPGS was necessary for stabilization of AmB-NPs suspension from aggregation in comparison to formulation without TPGS (Italia et al., 2011).

Increment of PVP from 1 to 4% has significant effect on MPS reduction and rise of release rate.

A simple, fast, accurate and reliable HPLC method for measuring AmB in *in vitro*. The developed analytical method was sensitive for AmB, could quantify as low as 250 ng/ml, and it capable for measurement of AmB concentration in all tested formulations. Under the assay conditions described, retention times for AmB and IS were around 2.87 ± 0.05 and 4.2 ± 0.09 min, respectively. The correlation coefficient was found to be ~0.998.

Chapter III. Pharmacokinetics of the Selected Oral Amphotericin-B Nanoparticles Loaded to PLGA-PEG Diblock Copolymer

3.1. Introduction

AmB is the gold standard treatment for sever systemic life-threatening fungal infections since 1959 (Bekersky et al., 1999, Cifani et al., 2012). It is currently used as the secondary treatment for leishmaniasis, with a 97% cure rate with no reported resistance (Wasan et al., 2009b). It is currently used as the secondary treatment for leishmaniasis, with a 97% cure rate with no reported resistance (Wasan et al., 2009b). Unfortunately, AmB is available in the market only for parenteral administration.

AmB state (monomers or aggregates) affects its efficacy and toxicity, since monomers forms are responsible for the therapeutic activity, while the aggregates forms are accountable for drug toxicity (Nishi et al., 2007).

Nephrotoxicity is the most serious chronic adverse effect of AmB; the serum creatinine concentration increases in more than 80% of patients receiving this drug (Sachs-Barrable et al., 2008, Tonomura et al., 2009).

AmB has an unknown metabolism and tissue distribution (Egger et al., 2001). The primary route of its elimination is not known (Drew, 2013).

The amphipathic nature of AmB significantly reduces its solubility in water and most organic solvents. Its aqueous solubility was improved by adding sodium deoxycholate to its formulation to produce a colloidal dispersion after reconstitution for intermittent intravenous (iv) infusion (Fungizone®). Severe side effects are associated with the administration of Fungizone®, such as nephrotoxicity, have restricted its clinical uses (Burgess and Birchall, 1972, Chuealee et al., 2011).

The development of parenteral AmB lipid-based formulations, Abelcet®, Ambisome® and Amphocil®, which have shorter course of therapy (3–5 days), highly effective and exhibit lower toxicity when compared to Fungizone® (Clements Jr and Peacock Jr, 1990, Baginski et al., 2005, Torrado et al., 2008). Regrettably, their cost is a great barrier to their common use (Sachs-Barrable et al., 2008, Falamarzian and Lavasanifar, 2010a).

AmB is also characterized by instability at gastric pH and is unable to cross the mucosal barrier of the GI to blood stream. The critical need for an oral drug delivery of AmB has steered several endeavors found in the literature about the development of AmB oral delivery systems to improve its antifungal activity and toxicity. These include

formulating AmB as nanosuspension (Kayser et al., 2003), as Poly(lactide-co-glycolide) nanoparticles (NP) employing vitamin E-TPGS as a stabilizer (Italia et al., 2009, Italia et al., 2011), as lipid-based oral formulation using Peceol (Sachs-Barrable et al., 2008) or as liquid antisolvent precipitation NP (Zu et al., 2014). Furthermore, AmB was loaded to Peceol and PEG-phospholipids (iCo-009) (Wasan et al., 2009a, Gershkovich et al., 2010, Sivak et al., 2011), to carbon nanotubes (Prajapati et al., 2011b), to gelatin coated lipid NP (Jain et al., 2012), to Chitosan–EDTA conjugate (Singh et al., 2013) or to Cubosomes lipid-based delivery (Yang et al., 2012, Yang et al., 2014). The latest formulations were AmB liposomes containing ceramides (Skiba-Lahiani et al., 2015) and AmB encapsulated with chitosan derivative (Serrano et al., 2015). These oral drug delivery were developed to enhance the solubility and gastrointestinal permeability of AmB, but none of them has been introduced to the market yet (Heurtault et al., 2003, Thornton and Wasan, 2009, Wasan et al., 2009a, Ibrahim et al., 2012, Yang et al., 2012).

The addition of glycyrrhizic acid (GA) as absorption enhancer was evaluated. GA is a major constituent of licorice with steroid-like, anti-allergic and antiviral activities (Pompei et al., 1980). It has been used orally as a sweetener and component of oriental medicines (Imai et al., 1999, Anand et al., 2010). In the field of drug delivery, GA is reported as a low-toxic oral absorption enhancer to improve the oral bioavailability of different drugs(Radwan and Aboul-Enein, 2002, Chen, 2009, Yang et al., 2015).

It was assumed that loading AmB to PLGA-PEG NP would improve its solubility and decrease its toxicity since the drug would be slowly released from the delivery system. It could also prevent AmB aggregation and thereby decrease its toxicity toward mammalian cells, while maintaining it in a monomeric form that favors antifungal activity (Brajtburg and Bolard, 1996, Torrado et al., 2008).

Lately, two published reports on the use of PLGA-PEG NP as AmB parenteral delivery systems. The first study was concerning about delivering AmB as a mannose-anchored engineered nanoparticulate for macrophage targeting (Nahar and Jain, 2009), while the second paper encapsulated AmB in PLGA-PEG NP to increase AmB solubility and to target the macrophage of infected tissues during visceral leishmaniasis (Kumar et al., 2015). To our knowledge, no published data about the development of AmB loaded to

PLGA-PEG NP for oral AmB delivery other than mentioned in chapter II. The in vitro studies of the developed formulations have indicated promising oral drug delivery system with acceptable drug content and dissolution rate. Therefore, after physical characterization of the developed selected PLGA-PEG NP formulations, the aim of this study was to confirm the practicability, safety and efficacy of using these novel AmB formulations as oral AmB delivery system in rats. The objective of this part of the study was to carry out pharmacokinetics screening of these formulations after iv and oral administrations to rats. The feasibility of glycyrrhizic acid as an absorption enhancer to improve AmB bioavailability was part of the investigation.

Several HPLC, reverse phase methods for AmB have been described in serum, plasma, tissue (Granich et al., 1986, Bekersky et al., 1996, Polikandritou Lambros et al., 1996, Echevarría et al., 1998, Zhan-rui et al., 2009, Chakrabarty and Pal, 2011). Table 3.1 list the HPLC assays published in the literature for the analysis of AmB in biological samples with the corresponding references. Proein precipitation (deproteinization) were the mostly utilized method for extraction, where C18 and acetonitrile were the most commenly used column and solvemt respectively. UV Dectection were manliy at 382 and 405 nm.

Advanced hyphenated technique such as liquid chromatographic tandem mass spectrometry (LC MS/MS) as shown in Table 3.2, is the revolution technique of traditional HPLC assays for determination of AmB in biological samples. Generally, the use of MS/MS as the detector has demonstrated better selectivity and sensitivity than the use of ultraviolet detector of conventional HPLC methods. It overcomes the effect of the yellow components in plasma, such as bilirubin (Xiong et al., 2009). These HPLC methods show either lake of sensitivity with minimum detection limits of 2 μg/ml in plasma (Xiong et al., 2009, Deshpande et al., 2010) or take longer run time >5.0 min, or use too many steps of clean up in extraction (Lee et al., 2001). Although the LC MS/MS techniques are characterized by a faster run time, to our knowledge no published data is reporting an assay of AmB using LC MS/MS with retention time < 3.5 min, with simple and efficient extraction technique. The present study was undertaken to develop a fast, robust, selective, sensitive, and precise LC MS/MS method for the monitoring of AmB in rats plasma using clopidogrel as IS.

Table 3.1. The literature reported HPLC for amphotericin B determinations in biological samples

Analytical technique	IS	Column	Mobile phase	Detection (UV-nm)	Retensiontime (min)	References
Deproteinization	-	CLC-trime-thylsilyl	acetonitrile :10 mM acetate buffer (pH 7.4)(40:60, v/v)	382	~ 7.8	(Hosotsubo et al., 1988)
Solid-phase extraction	N-acetylamphoteri-cin B	C_{18}	acetonitrile : 2.5 mM Na, EDTA (45:55, v/v)	382	6.76	(Wang et al., 1992)
Deproteinization	piroxicam	Ultrabase-C	acetonitrile: acetic acid (10%) : water (41:43:16, v/v/v)	405	5.7-5.9	(Echevarría et al., 1998)
Deproteinization	1-amino- 4-nitronaphthalene		acetonitrile : 10mM sodium acetate buffer(pH 4) (11:17, v/v)	408		(Otsubo et al., 1999)
Solid-phase extraction	-	LiChrosorb-RP-8	acetonitrile:methanol:0.010 M NaH_2PO_4 buffer (41:10:49, v/v/v)	405	6.7	(Egger et al., 2001)
Deproteinization	1-amino-4-nitronaphtalene	Bondapak C-18	acetonitrile-disodium EDTA (20 mM) (45:55, v/v pH 5)	382	-	(Eldem et al., 2001)
Solid-phase extraction	1-amino-4-nitronaphtalene	Sep-Pak C-18	methanol: 11 mM phosphate buffer (pH 7.4) (40:60, v/v)	407	-	(Eldem et al., 2001)
Deproteinization	natamycin	Reverse phase column	acetonitrile: 0.05 N sodium acetate (34:66, v/v)	405	-	(Bekersky et al., 2002a)
Deproteinization	1-amino-4-nitronaphthalene		acetonitrile : 10mM sodium acetate buffer, pH 4.0 (11:17, v/v)	408		(Otsubo et al., 1999, Fukui et al., 2003a)
Deproteinization	-	Econosphere C18	acetonitrile: 0.02 M EDTA (55:45, pH 4.5)	405	-	(Malone et al., 2013)
Deproteinization		ODS-Hypersil C18	acetonitrile :2.5 mM EDTA (45:55, v/v)	382	4.7	(Manosroi et al., 2004)
Deproteinization	-	Lichrosorb® RP-8	-	405	-	(Brime et al., 2003)
Deproteinization	p-nitroaniline	Sep-Pak Vac	10 mM sodium acetate buffer with	405	-	(Hong et al., 2007)

		C18	10 mM EDTA (pH 3.6): acetonitrile (60:40)			
Deproteinization	1-amino-4-nitro naphthalene	Nucleosil® C18	acetonitrile: 10mM acetate buffer (pH 4)	407	6.8	(Italia et al., 2009)
Deproteinization	-	Luna C18	acetonitrile: tetrahydrofuran: o-phosphoric acid (60:30:10, v/v/v pH 6.0)	287	7.72	(Balamuralikrishna and Syamasundar*, 2010)
Deproteinization	-	BDS Hypersil C18	acetonitrile: acetic acid: water (57:4.3:38.7, v/v/v)	407	15	(Wasan et al., 2010)
Deproteinization	Lornoxicam	Hypersil ODS C18	10 mmol phosphate buffer : acetonitrile (65:35, v/v)	407	5.2	(Chakrabarty and Pal, 2011)
Deproteinization	-	Phenomenex luna C18	organic phase methanol/ acetonitrile/tetrahydrofuran (41/18/10) to buffer (2.5mmol/L EDTA-2Na) (55/45, v/v)	383, 303	>15	(Eldem et al., 2001)
Deproteinization	1-amino-4-nitronaphthalene	BDS Hypersil C18	5 mM of sodium acetate at pH 4.2	225	-	(Ibrahim et al., 2013)
Deproteinization	4-nitro-1-naphthylamine	symmetry C18	acetonitrile : 0.1 M acetate buffer(pH 4.2)	405	10.8	(Malone et al., 2013)
Deproteinization	No IS	XBridgeTM C18	acetic acid (0.73%) : acetonitrile (60:40, v/v)	408	2.7	(Balabathula et al., 2013)
Deproteinization	-	Spheri-5, CYANO	10 mM sodium acetate (pH 4) : acetonitrile (72:28, v/v)	408	5.9	(Kumar et al., 2014)

Table 3.2. The literature reported LC MS/MS for amphotericin B determinations in biological samples using protein precipitations for extraction

IS	Column	Mobile phase	Detection (UV-nm)	Retension time (min)	References
Natamycin	Symmetry C18	methanol:water:acetic acid (69:29:2, v/v/v)	924 →906 m/z	-	(Bekersky et al., 2002b)
Natamycin	Phenomene x Synergi Max-RP	10 mM ammonium acetate (pH 3.6):acetonitrile (620:380)	924→ 906.5 m/z	-	(Hong et al., 2007)
Rifaximin	Sunfire C18 column	0.1% formic acid : methanol (0.1% formic acid) (40:60%, v/v)	924→ 906.6 → 743.4 m/z	4.3	(Xiong et al., 2009)
Natamycin	RP18e	mobile phase"A" 5mM ammonium acetate buffer (pH 6):Acetonitrile : methanol (48:20:32, v/v) mobile phase"B" 5mM ammonium acetate buffer (pH 6): acetic acid: acetonitrile: methanol (25:5:5:70, v/v/v/v)	924.5→ 906.6 m/z	5.1	(Deshpande et al., 2010)
Natamycin	Luna CN	3.5 mM ammonium acetate (pH 4): methanol (10:90, v/v)	922.7 →183.3 m/z	3.37	(Bhatta et al., 2011)

3.2. Materials and Apparatus

3.2.1. Materials

All material and chemicals are of analytical grade and used as received. Table 3.3 shows the list of materials and polymers, used in this study and their manufacturer, respectively.

Table 3.3. New chemicals used in this part of the work

Chemical	**Supplier**	**Address**
Amphotericin B (99.8% purity)	Sigma–Aldrich	St. Louis, MO, USA
Clopidogrel (98% purity)		
Glycyrrhizic acid		
Dimethylsulfoxide (DMSO)		
Triethylamin		
Formic acid- Mass grade		
Ammonium formate		
PLGA-PEG copolymers polymer	Boehringer Ingelheim	Ingelheim, Germany
Acetone	Fisher, Chemical	Fisher, UK
Dichloromethane		
Hydrochloric acid		
Methanol-HPLC grade		
Acetonitrile- HPLC grade		
Acetic acid		
Polyvinyl pyrrolidone (PVP)	BASF	Ludwigshafen, Germany
Miglyol®-812	Sasol	GmbH, Germany
Vitamin E (TPGS)	Peboc	Eastman, UK
Pluronic F68	Ruger Chemical	New Jersey, USA
Formic acid (Methanoic acid)	Avonchem	UK
Milli-Q Reagent Grade water system	Millipore Corporation	Bedford, MX, USA

3.2.2. Apparatus

Table 3.4. shows a list of the apparatus used during LC MS/MS analysis of AmB in biological samples.

Table 3.4. Apparatus used during analysis of Amphotericin B

Apparatus/components	**Purpose**	**Manufacturer**
Mettler Toledo-AG285 Sartorius –ME36S-MicroBalance	Analytical balance	Mettler, Greifensee, Switzerland -Sartorius AG, Goettingen, Germany
pH meter, Backman, Model 72	Measure pH	Beckman Instruments, Fullerton, USA
Waters Aquity UPLC™	LC MS/MS system	Waters Corp., Milford, USA
Aquity UPLC™ BEH C18 column 50mm x 2.1mm, 1.7 µm triple quadropole tandem-mass detector	Separation column – maintained at 40 °C	Waters Corp., Milford, USA
MassLynx™ V 4.1 software with TargetLynxTM V 4.1 program	Data recording and processing	Waters Corp., Milford, USA

3.3. Preparation of AmB-Loaded PLGA-PEG Copolymer

AmB loaded to PLGA-PEG was formulated according to previously mentioned in Chapter II with slight modification by adding GA. Diblock, 15% PEG RGPd 50155 copolymer formulation (C6) was selected as the base formulation for this part and is named F1. To investigate the feasibility of PLGA-PEG polymer versus non peglyated polymer, R 203 H, poly (D,L-lactide), AmB-NPs was prepared by the same technique. All formulations were prepared at least in triplicate.

When GA was added to F1 just prior to administration the formulation is called F2-GA-out-1, F2-GA-out-2 and F2-GA-out-3, where, 1,2 and 3 refer to the percentage of GA added. When GA 2% was added to the organic phase during F1 preparation, the resulted formulation is called F2-GA-in-2.

Doubling the amount of AmB in the formulation with addition of GA inside the formulation is called F3-GA-in-2, while F3-GA-out-2 is the same composition but the GA was added just prior to administration.

Table 3.5 shows the composition and method of *in vivo* administration of the formulations tested in rats.

Immediat0ly before administration, the specific weight of the lyophilized AmB-loaded PLGA-PEG NPs were dispersed in water solution either alone or in combination with GA as 1 and 2 %. The volume of the PO suspension dose was 1.0 ml.

Table 3.5. The composition and method of administration of the developed formulations used during the study

Animal Group	Route	Formulation	Carrier/ Polymer	Drug Amount, mg	GA % IN	GA % OUT	Dose mg /kg
I	PO	Fungizone®	Sodium deoxycholate	50	-	-	10
II	iv	F1	Diblock, PLGA-PEG (Lactic to glycolic acid ratio1:1 molar ratio)with 15% PEG; molecular weight 6000 Dalton RGPd 50155	20	-	-	1
III	PO	F1		20	-	-	10
IV	PO	F2-GA-out-1		20	-	1	10
V	PO	F2-GA-out-2		20	-	2	10
VI	PO	F2-GA-out-3		20	-	3	10
VII	PO	F2-GA-in-2		20	2	-	10
VIII	PO	F3-GA-in-2		40	2	-	10
IX	PO	F3-GA-out-2		40	-	2	10
X	PO	Non PEG	Poly (D,L-lactide)Monoblock R 203 H	40	-	-	10

3.4. Animals Dosing and Blood sampling

All the experiments were performed in accordance to the ethical guidelines established and approved by the committee on the use and care of laboratory animals at King Saud University. Sixty albino Sprague-Dawley rats (353.17 ± 26.61 gm) were provided from the animal house of King Saud University. Animals were housed in polypropylene cages in an animal facility with a 12 h light-dark cycle and controlled temperature and humidity. The rats were given free access to water ad libitum and fasten for 10 h before the study. Standard rat chow was given after 2 h of dosing for the duration of the study. The rats were randomly divided into ten groups (I –X, n=6) and were dosed according to Table 3.5.

After dosing, blood samples (600 µl) were withdrawn from the venous-orbital plexus from each rat in heparinized tubes. Blood samples from the same rats were collected at 0, 1, 3, 6, 12 and 0.5, 2, 4, 8, 24 h after drug administration in two different occasions, separated by 3 weeks. The animals were slightly anesthetized with carbon dioxide during blood sampling. Plasma samples were separated by centrifugation at 4000 rpm for 15 min and were stored at -20^{o}C prior to drug assay.

3.5. LC MS/MS Chromatographic system and conditions

Analysis was carried out on a Waters Acquity UPLC^{TM} system with the components listed in Table 3.6. For chromatographic conditions' optimization, different mobile phases were examined at different pH using a variety of columns. AmB has two pKa values, a basic pKa (8.12) due to amino group at position 48 and acidic pka (3.72) due to an acidic group at position 15 (Figure 1.25). Therefore, a pH of 3.0±0.2, was suitable for keeping AmB in one ionic state for mass analysis. Ammonium formate was selected to avoid overloading of ionization source with buffer (Deshpande et al., 2010). The gradient elution for LC MS/MS analysis consisted of two solvent compositions: Solvent A: Methanol: Acetonitrile (50:50, v/v) containing 0.1% formic acid and solvent B: 10 mM ammonium formate (pH 3±0.2), containing 0.2% formic acid and 1% acetonitrile. The gradient began with 35.0% eluent A and increased to 90% and over 2.0 min prior to return to 35.0% A at 2.7 min. Throughout the process, the flow rate was set at 0.3 ml/min at the start of the run

and increased to 0.4 ml/min within 2 min, returning to 0.3 ml/min at 2.7 min. The total run time was 3.2 min. Data were collected in multi-channel analysis (MCA) mode.

The LC MS/MS was connected to a triple quadropole tandem-mass detector, with an electrometry ionization (ESI) source for mass spectroscopy detection. The ESI source was set in positive ion mode and quantification was performed using multi-reaction monitoring (MRM) mode for the most suitable mass transitions. The optimal mass spectrometry parameters are listed in Table 3.6 Most prominent and stable fragments for AmB and internal standard (IS), with scan time of 0.10 s per transition. The optimized collision energy for AmB and IS were 15 and 37 eV, respectively.

Table 3.6. Setting for LC MS/MS detection of Amphotericin B

Source (ESI +) and Analyzer	**Settings**
Capillary voltage (kv)	3.5
Cone voltage (v)	15
Extractor (v)	2.0
Radio frequency lens (v)	0.10
Source temperature (C)	115
Desolvation temperature (C)	400
Cone gas flow (L/h)	6
Desolvation gas flow (L/h)	800
Collision energy (eV)	15
Collision gas flow (ml/min)	0.25

3.6. Preparation of Amphotericin B Standards and Quality Control

All solutions of AmB were stored in polypropylene vials to minimize adsorption of AmB to glass (Lee et al., 2001). Stock standard solution of AmB was prepared in methanol: DMSO (9:1, v/v) at a concentration of 0.2 mg/ml and stored in 4.0 ml plastic vials at −20 ^{o}C covered with aluminum foil to protect AmB from light. For IS stock solution, 5.0 mg was dissolved in 10 ml methanol and stored at −20 ^{o}C in 3 of 4 mL amber vials. Different working standard solutions of AmB (100-4000 ng/ml) and the IS

(1.5µg/ml) were prepared by dilution of the above mentioned stock solutions with pure methanol and were kept at −20 ^{o}C.

3.6.1. Plasma Sample Preparation

The plasma calibrations standards were prepared in six replicates at different concentrations between 100 - 4000 ng/ml in a 1.8 ml Eppendorf tube. Blank Rat plasma was withdrawn from healthy rats by cardiac puncture in the laboratory. This technique were done in the presence of a veterinary technician who will be able to assess that animals are handled properly and are not subjected to unnecessary pain and/or distress. Blank rat plasma (250 µl) was spiked with aliquots of AmB working solutions and 20 µl (30 ng) of the IS. Low, medium and high concentration quality control (QC) samples at concentrations of 100, 1000 and 4000 ng/ml of AmB with 120 ng/ml of IS. The prepared plasma samples were subjected to protein precipitation by the addition of 1 ml of methanol; the mixture was vortexed at high speed for 1 min followed by centrifugation at 20,000 rpm for 15 min at 10 ^{0}C. The supernatant was transferred into a clean 5 ml Pyrex glass tube and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 150 µl of 90% methanol solution. After vortexing for 1 min, centrifuging at 4000 rpm for 4.0 min, and transferring into a plastic autosampler vial with pre-slit septum (Waters, USA) 2.0 µl were injected into the LC MS/MS system for analysis.

3.6.2. Ion Suppression Study

The absence of ion suppression was demonstrated by the method of Matuszewski et al. (1998). Six different formulations of drug-free and IS-free rat's plasma were extracted. The extracts were reconstituted with AmB at three nominal concentrations 100, 1000, and 4000 ng/ml (low, medium, high). The peak areas of the samples were compared to that of the unextracted reference standard solutions containing the equivalent nominal amount of AmB in the mobile phase (n = 6). The mean area ratios (reconstituted extracts/reference solutions) were 0.95 for AmB with R.S.D. of < 3.9%. Thus, no ion-suppression was observed.

3.7. Method Validation

Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites are critical for the successful conduct of preclinical and/or biopharmaceutical and clinical studies. Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analyte in a given biological matrix, such as blood, plasma, serum or urine, is reliable and reproducible for the intended use. The developed method was validated in regards of selectivity, linearity, precision, accuracy, carry over, extraction recovery and stability in accordance with FDA guidelines and United State Pharmacopeia XXX III (Administration, 2007).

The selectivity of an analytical method is its ability to measure accurately an analyte in the presence of endogenous compounds. Therefore, six blank, drug-free, plasma samples obtained from six rats were analyzed according to the procedure described above. The corresponding chromatograms were tested for possible interferences at the retention times of AmB and the IS.

The specificity of the method was investigated by comparing the chromatogram of blank plasma spiked with standard solutions to the samples collected from rats after AmB administration.

Intra-day precision and accuracy were determined within one day by analyzing six replicates of the QC samples at concentrations of 100, 1000, and 4000ng/ml of AmB. The inter-day precision and accuracy were determined on three separate days. The intra- and inter-day precision were defined as the relative standard deviation (R.S.D. %). The accuracy was presented as percent relative error, R.E. [(measured concentration−nominal spiked concentration)/ nominal spiked concentration] ×100. Extraction efficiency was determined for the QC samples at the three concentrations levels (low, medium, high) with those of post-extraction spiked blank plasma samples for AmB concentrations. The absolute extraction recoveries were calculated by comparing the peak areas of the samples to that of the unextracted standard solutions containing the equivalent amount of AmB (n = 6). The nominal value of AmB concentration (ng/ml) in plasma was plotted as a function

of the peak area ratio obtained of AmB and the IS. The day curve was accepted, if the R.S.D. % was <20% for all the tested concentrations (low, medium and high).

The limit of detection (LOD) was defined as the lowest concentration of the analyte resulting in a signal-to-noise (S: N) ratio of >5:1. The lower limit of quantitation (LLOQ) was defined as the lowest drug concentration of the analyte resulting in an S: N ratio >10 which can be determined with a R.S.D. <20% and an accuracy of 100±20% on a day-to-day basis.

Accuracy and precision at the LLOQ were estimated. The robustness of the method was determined, by using two different Acquity HUPLC^{TM}BEH RP_{18}, 1.7µm column, 2.1 mm x 50 mm (Waters Corp, Milford, MA, USA).

3.8. Stability

Drug stability in a biological fluid can be affected by the storage conditions, the chemical properties of the drug and the container used. Stability measures should assess the stability of the analytes throughout sample collection and management, after short-term in autosampler waiting for analysis, and after going through freeze and thaw cycles during the analytical process. Stock solutions of the analyte for stability assessment should be prepared in an appropriate solvent at known concentrations.

Freeze–thaw stability of the plasma samples were evaluated by exposing plasma extracted samples to freeze (−20 ^{0}C) – thaw (room temperature) cycles immediately after reconstitution, after 0, 3,7 and 14 days of storage at -20 ^{0}C of the processed samples. The stability of the processed samples in autosampler was also evaluated by keeping the sealed, with parafilm, samples in the autosampler condition at 10 ^{0}C for 72 h. The samples were analyzed at 0, 24, 48 and 72 h.

AmB was also subjected to a drastic conditions, in amber volumetric glass by diluting it in water, 1M solution of NaOH and 2N HCL solution (n=6) and AmB area under the peak (AUP) was measured as the zero AUP. Each solution was carefully heated to boiling and left to cool down and the AUP of AmB after boiling was measured.

3.9. Data and Statistical Analysis

All data in this study were expressed either as the mean ± SD of six replicates for the assay or seven replicates for the rats study. The standard curves were calculated by linear regression without weighting, using the equation Y = 0.0007X – 0.127, where Y is the AUP ratio of the drug to the IS, – 0.127 is the intercept, 0.0007 is the slope, and X is AmB concentration. The RSD was calculated for all values.

The plasma AmB concentration vs time data of the rats were analyzed using a model-independent method for the main purpose of this study (Gibaldi and Perrier, 1982, Preacher et al., 2006). The mean maximum concentration (C_{max}) and the time to reach C_{max} (T_{max}) were derived directly from the individual plasma levels.

The elimination rate constant (k) was calculated from the slope of the regression line that best fit the terminal part (last three or four points) of the log–linear concentration–time profile of AmB. The terminal half-life ($t_{1/2}$) was calculated from 0.693/k. The area under the curve from time 0 to 24 h (AUC0–24) was estimated by linear trapezoidal rule and was extrapolated to time infinity (AUC) by the addition of C_n/k where, C_n is concentration of the last measured plasma sample. The total body clearance (Cl) was estimated using the equation Cl=dose/AUC. The volume of distribution (V) was determined from the equation k = Cl/V. For oral data, the absorption rate constant k_a was estimated by the method of residual(Tozer and Rowland, 2006). The absolute bioavailability (F) was calculated by the equation (AUC_{po}/$Dose_{po}$) / (AUC_{iv}/$Dose_{iv}$) after the iv of F1 and oral administration of the tested formulations, while the relative bioavailability (F_{rel}) was calculated using ($AUC_{PO\ formulation}$ / ($AUC_{F1\text{-}PO}$) or $F_{rel\ to\ Fungizone}$ using ($AUC_{PO\ formulation}$ / ($AUC_{Fungizone\text{-}PO}$).

The student t-test was used to examine the concentration difference at each day, and one-way analysis of variance (ANOVA) was employed to evaluate the reproducibility of the assay. Other tests were used using IBM SPSS Statistics 21. A P value < 0.05 was considered significant.

All statistical differences in data were evaluated using IBM SPSS Statistics 21. The Student t-test was used to examine the concentration difference at each day and one-way analysis of variance (ANOVA) was employed to assess the reproducibility of the assay and other tests were used when needed. P< 0.05 was considered significant.

3.10. Results and Discussion

3.10.1. Liquid Chromatography Assay of AmB

Mass spectroscopy (MS) parameters were optimized for selectivity and sensitivity in positive ion mode. Both the analyte and internal standard (IS) have ability to accept protons and generate [M+H]+ ions. Protonated parent ions for AmB and IS quantification were achieved using MRM of the transitions of m/z 925.1 ± 1.2→742.3 ± 0.9 for AmB and m/z 321.8 ±0.5 →155 ±0.6 for IS (Figure3.1).

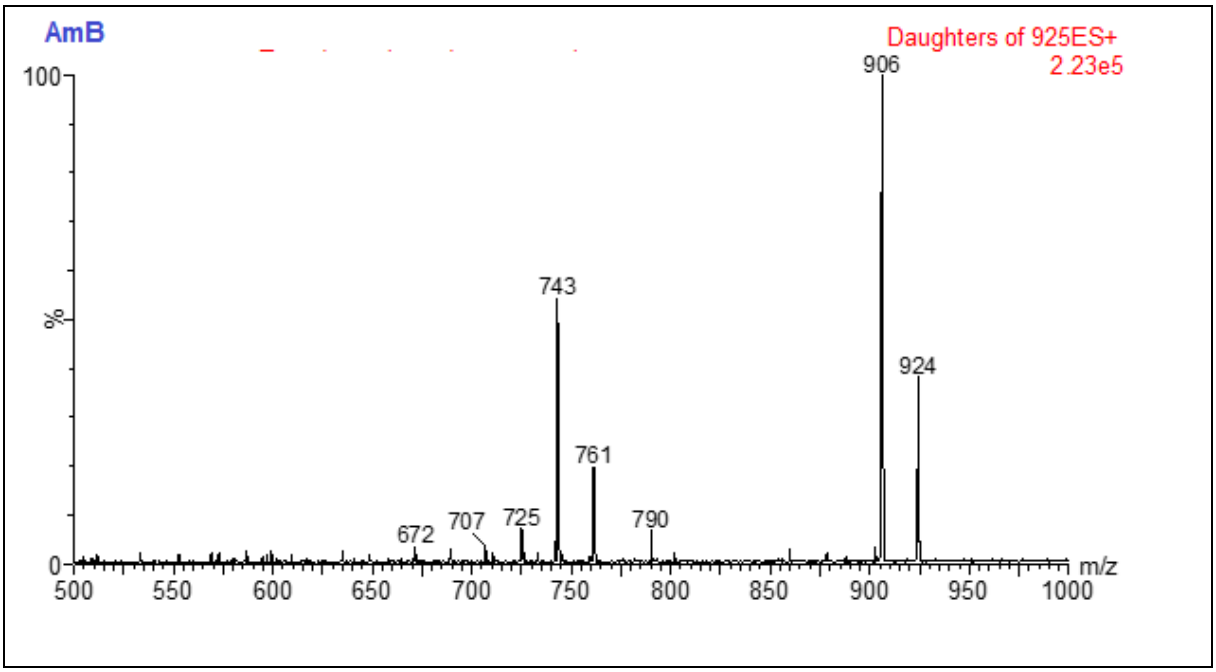


Figure 3.1. MS/MS ion spectra fragments of amphotericin B

Figure 3.2 is a representative chromatograms of AmB and IS after extraction of plasma samples. The chromatogram of blank rats plasma is shown in Figure 3.2, A1 and the MRM of AmB and IS are in A2; the MRM blank rat plasma spiked with the AmB at the LLOQ (100 ng/ml) and IS are shown in Figure 3.2 as B1 and B2 respectively The retention times of AmB and IS are 1.7 ±0.05 and 2.1± 0.04 min, respectively, showing proper separation. The assay run time was 3.2 min which is the shortest among AmB published separation assays (Xiong et al., 2009, Deshpande et al., 2010). During the 7 months of

validation, there was no significant change (P= 0.299) in the observed retention time of AmB or the IS (R.S.D. < 0.4 %).

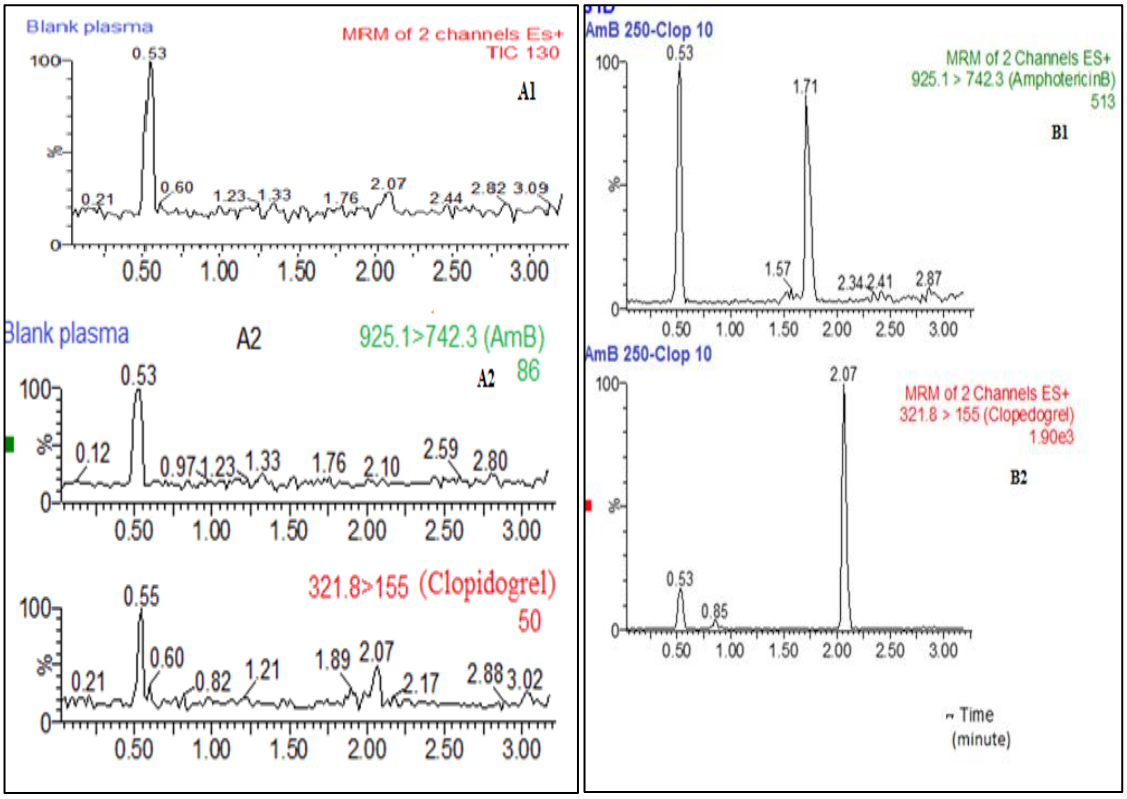


Figure 3.2.MRM transition of extracted chromatograms of blank rat plasma and its transition (A1 and A2) MRM transition of extracted chromatograms of standard spiked AmB in rat plasma at 250 ng/ml and IS and its transition (B1 and B2)

Figure 3.2 is an indication of no endogenous plasma components eluted at the retention times of either AmB or the IS in any of the spiked blank plasma samples collected from different rats or pooled rats' plasma, which proves the assay specificity.

Excellent separation between AmB and IS, minimal background baseline noise was obtained as shown in Figures 3.2. It was also observed that the representative chromatogram of rat plasma samples showed similar chromatographic behavior to spike or quality control samples which is another proof of good assay selectivity.

The LOD of this assay was 50 ng/ml in rats plasma with the corresponding R.S.D. % of about 20 % at a signal-to-noise ratio of >5 while, the LLOQ was 100 ng/ml at a signal-to-noise ratio of >10, with the corresponding R.S.D. of about 14 % at injection volume of 2.0 µl. The peaks of AmB and IS are well resolved and LLOQ was sensitive enough to detect AmB at all sample time points used.

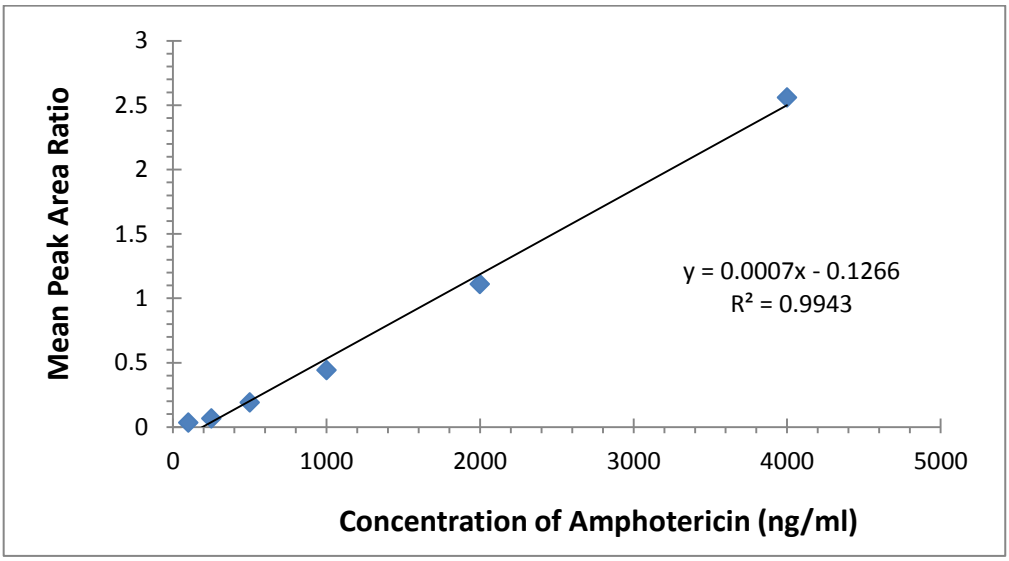


Figure 3.3. Standard calibration curve of amphotericin B

Excellent linear relationships (Y=0.0007X - 0.12) with R^2> 0.994 (Figure 3.3) was demonstrated between AUP ratio of AmB to the IS in rats plasma over the studied concentration ranges. The mean R^2 was > 0.99 and the R.S.D. % of the slopes of the three standard plots was <13.3%. Analysis of variance of the data (n=18) indicated no significant difference (P > 0.05) in the slopes, intra- and inter-day, of the calibration curves. The results confirmed the reproducibility of the assay method.

The mean percentage recovery of AmB was 93.7 ± 7.2 with R.S.D. of ≤ 10%. There was no significant difference (P > 0.05) in the extraction efficacy of the present assay over the range of concentrations studied. The accuracy and precision results are shown in Table 3.7. Precision is represented as R.S.D. % and accuracy was calculated as relative error (R.E. %). The intra-run and inter-run precision (R.S.D. %) was <13% and accuracy

as R.E. was < 8%. There was no evidence of sample carry-over from run to run. In addition, the matrix effect assessed by spiking samples post-processing showed R.S.D. < 8% difference from spiked injection solvent.

Table 3.7. Inter- and Intra-day statistics

Measured concentration (ng/ml)	Day 1	98	970	4126
		106	991	3974
		110	955	4140
		97	985	3960
		111	1010	4148
		99	970	3874
	Day 2	111	980	4100
		101	992	3970
		94	900	4020
		98	1250	3992
		103	1130	3900
		105	980	4080
	Day 3	99	1100	3950
		101	1050	4020
		106	1040	4150
		110	1045	4090
		107	995	4020
		98	980	3950
Intra-day (on day3)	**N**	6	6	6
	Mean	103	1017.9	4025.8
	SD	5.4	78.7	86.7
	Accuracy (RSD %)	5.2	7.7	2.1
	Recovery (%)	97	100.7	100.7
	Recovery (RSD %)	8.2	7.3	5.2
Inter-day statistics	**n**	6	6	6
	Mean	103.5	1035	4030
	SD	4.85	42.9	78.7
	Accuracy (RSD %)	4.7	4.1	1.9
	Recovery (%)	97.8	100.4	99.7
	Recovery (RSD %)	7.3	9.8	7.8

Figure 3.4 shows that AmB was stable in the processed samples held in the autosampler at 10 ^{0}C for 24 h with mean calculated values within 6.7% of the nominal concentration. However, the samples lost 67.3% (R.S.D. of 7.9%) of its nominal concentration within three days if stored protected from light in the autosampler. Therefore, it is not recommended to keep AmB in the autosampler more than overnight analysis to ensure reproducibility of the assay. The freeze-thaw temperature cycles did not significantly (P>0.05) affect the stability of AmB in first cycle, after 72 h, with the mean calculated values within 2.3% of the nominal concentration. While after the third cycle, 42% of AmB was lost with R.S.D. of 5.6%. Unexpectedly, exposing AmB to drastic conditions revealed that AmB is stable in 2N HCl solution even after boiling, losing only 5.7% (R.S.D. of 7.3%) of its nominal value. In the meantime, both water and 1N NaOH showed complete loss of AmB even without boiling for all tested samples (n=6). Therefore, the use of small volume of 2N HCl (200 µL) to solubilize it in the preparation of its formulations was not significant to affect its stability as was mentioned in literature of its instability in acidic medium.

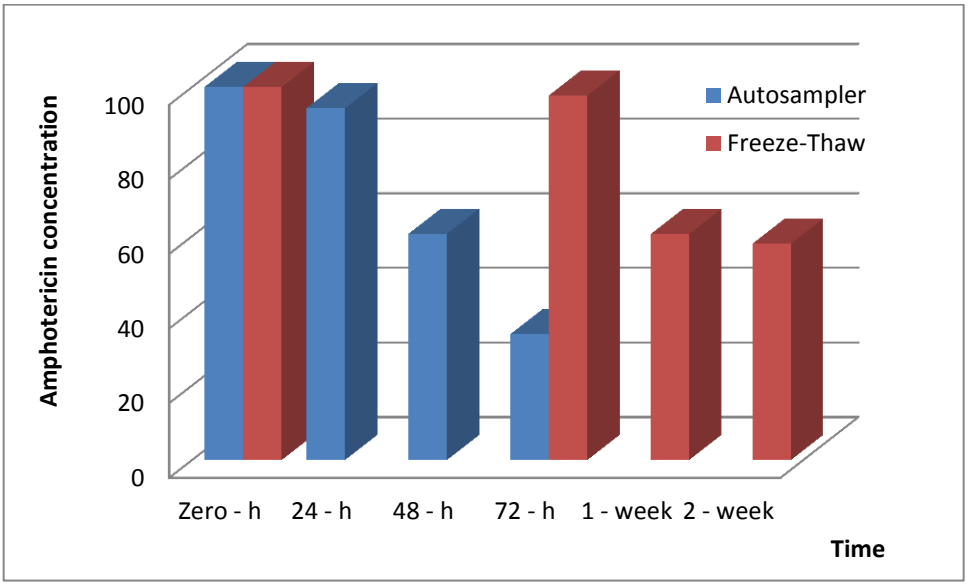


Figure 3.4. Stability of Amphotericin in autosampler and after freezing –thawing cycles

3.10.2. Amphotericin-B Pharmacokinetics in Rats

Novel oral biodegradable stealth polymeric nanoparticles of AmB have been successfully fabricated by an emulsification diffusion method using PLGA-PEG Diblock copolymer. Drug loaded to PLGA-PEG NPs has been previously *in vitro* characterized in Chapter II. Rats were used in this study since it is an appropriate animal model to investigate the GI absorption due to the similarities in intestinal characteristics between rats and humans (Risovic et al., 2003).

The hypothesis that loading AmB to PLGA-PEG diblock copolymer would significantly improve its GI tract absorption without increasing its induction of nephrotoxicity was the driving force of developing AmB oral formulation.

Nowadays, PLGA-PEG and PLA-PEG-PLA were being used to improve the release rate of different classes of drugs from NPs systems intended for both parenteral (Allémann et al., 1998, Huh et al., 2003, Packhaeuser et al., 2004, Cheng et al., 2007, Saadati and Dadashzadeh, 2014, Teekamp et al., 2015) and oral administrations (Yoo and Park, 2001, Garinot et al., 2007, Fernandez-Carballido et al., 2008, Khalil et al., 2013, Yan et al., 2015).

Figure 3.5 shows the semi-logarithmic mean plasma concentration–time profiles of AmB following F1 formulation, single dose study, given as 1.0 mg/kg (F1-iv) and 10 mg/kg (F1-PO) to two different groups of rats via iv and PO administrations, respectively. Fungizone®, 10 mg/kg was given to the third group of rats via PO administrations (Fungizone®-PO) which was used for comparison. After iv administration, a two compartment open model seems adequately describes the kinetics of AmB in rat plasma with a fast distribution, over the first 4 h, followed by slow elimination. A similar disposition model of AmB was previously described in rats after iv and PO administrations (Robbie, 1998, Echevarría et al., 2000). However, a model-independent method was used instead for the main purpose of this study and for simplicity (Eldem et al., 2001, Brime et al., 2003, Gershkovich et al., 2009). The pharmacokinetic parameters of AmB after the administrations of F1-iv, F1-PO and Fungizone®-PO in rats are summarized in Table 3.8.

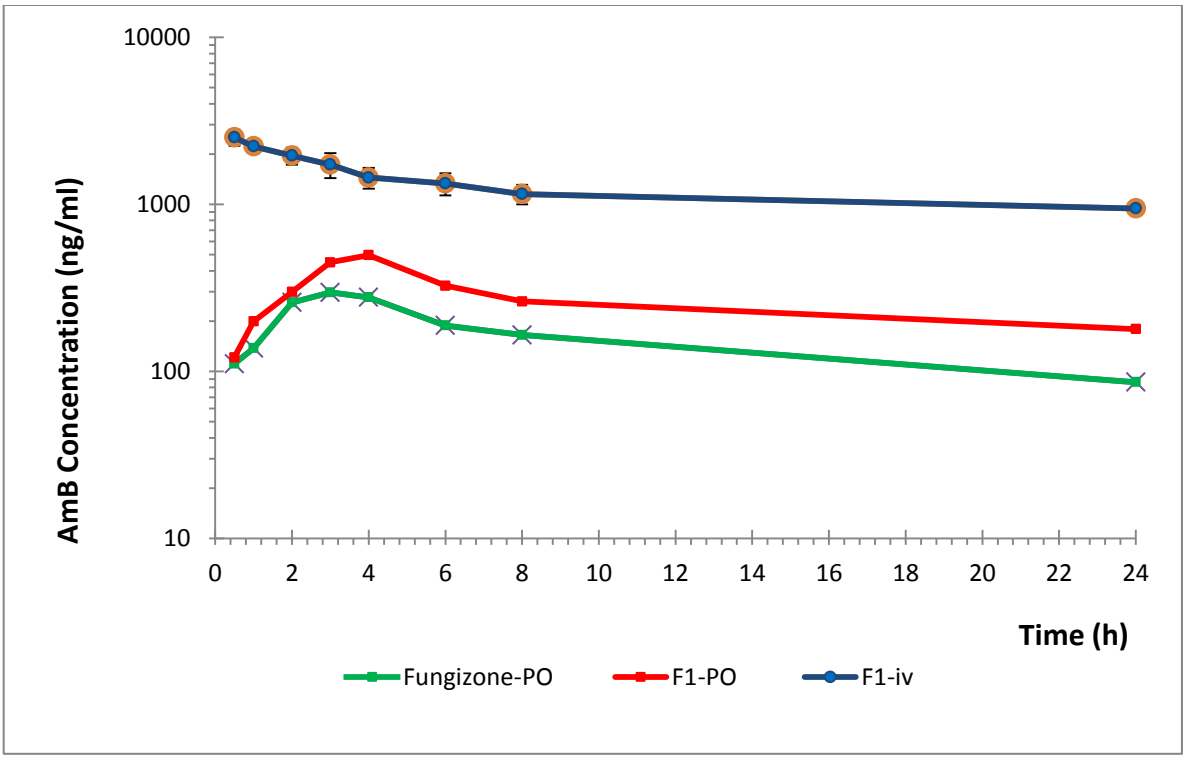


Figure 3.5. Semi-logarithmic plasma AmB concentration-time profiles (mean ± SD) after intravenous administration of Fungizone® and F1-iv at dose of 1.0 mg/kg and after oral administration of C6-PO at dose of 10 mg/kg to rats (n=6).

Table 3.8. Pharmacokinetic parameters of Amphotericin B after iv and oral administrations of AmB -Loaded PLGA-PEG formulations and Fungizone in rats (n=6)

Tested Parameters	**F1-iv**	**F1-PO**	**Fungizone®-PO**
Dose, mg/kg	1	10	10
C_{max}, ng/mL	-	480.2± 38.7	298.2± 28
$AUC_{0-\infty}$ (µg.h/l)	84211.2 ± 33935	12325.1 ± 1511.3	9836.3 ± 1654.1
k (h^{-1})	0.0208 ± 0.0084	0.0298 ± 0.0060	0.0019 ± 0.0015
Cl (ml/h/kg)	13.8 ± 6.1		
$t_{1/2}$ (h)	38.1 ± 14.8	24.0 ± 4.4	35.3 ± 2.6
V (ml/kg)	666.1 ± 109.7		
F	-	0.0.0145	0.0115
$F_{rel\ Fungizone}$	-	1.25	

After iv administration of F1-iv, AmB shows a relatively long elimination $t_{1/2}$ ranged from one to two days in rats due to its low Cl (8 to 23.2 ml/h/kg) and Large apparent volume of distribution in the range of 552.7 to 806.3 ml/kg.Although the iv dose was one tenth the PO doses of either F1-PO or Fungizone®-PO, F1-iv shows a pronounced higher plasma concentrations of AmB ($P<0.05$). This could be attributed to either incomplete absorption of AmB or the drug is subjected to a significant first pass in the liver. A statistically significant higher C_{max} of AmB was observed following F1-PO than that after following Fungizone®-PO administrations. The observed mean plasma $AUC_{0-24h}$and $AUC_{0-\infty}$ afterF1-PO were about 63.7% and 36.4%, respectively, higher than that of Fungizone®-PO. The bioavailability (F) of F1-PO was 36.4% higher than that of Funzizone®-PO due to the improvement of AmB release when loaded to PLGA-PEG diblock but the results were not satisfactory.

Several literatures show that AmB polymeric nanoparticles formulations, have proved antifungal efficacy and lower toxicity with increase accessibility of the drug to organs and targeted tissue (Amaral et al., 2009, Laniado-Laborín and Cabrales-Vargas, 2009, Souza et al., 2015). It was also reported that some carriers added during the formulation process affect the aggregation state and hence AMB activity(Legrand et al., 1992). Barwicz etal. have demonstrated the ability of surfactants to reduce the toxicity of AMB via decreasing the aggregation state of the drug (Barwicz et al., 1992, Adams and Kwon, 2003).

Currently, PLGA and PLGA-PEG are being used to improve the release rate of different classes of drugs from NPs systems intended for both parenteral (Allémann et al., 1998, Huh et al., 2003, Packhaeuser et al., 2004, Cheng et al., 2007, Saadati and Dadashzadeh, 2014, Teekamp et al., 2015) and oral administrations (Yoo and Park, 2001, Garinot et al., 2007, Fernandez-Carballido et al., 2008, Khalil et al., 2013, Yan et al., 2015).The ability of PLGA-PEG to enhance bioavailability of hydrophobic drugs, have been demonstrated for different classes of drugs. Curcumin potential efficacy is limited by its lack of solubility in aqueous solvents and poor oral bioavailability. PLGA–PEG NPs were able to increase curcumin bioavailability by 3.5-fold compared to curcumin loaded PLGA NPs alone (Anand et al., 2010). While insulin-loaded PLGA–PEG NPs show pH sensitivity. There were large extent of insulin released from NPs in intestine and fewer in

gastric; thus, copolymer has ability to protect insulin from enzymes in the gastric environment (Hosseininasab et al., 2014).Moreover, loading AmB to non PEGylated polymer (poly(D,L-lactide), R 203 H), showed no absorption of AmB at all time points. This is a confirmation of the importance of the PEG in PLGA polymer to promote the absorption of AmB after PO administration.

GA and its derivatives have been utilized in enhancing drug absorption (Tanaka et al., 1992, Anand et al., 2010, Imai et al., 1999, Cho et al., 2004, Radwan and Aboul-Enein, 2002). Significant enhanced rectal absorption of AmB suppositories in rabbits following GA addition in comparison to formulation containing no GA (Tanaka et al., 1992, Anand et al., 2010). Insulin nasal spray formulations for nasal delivery containing GA showed improve bioavailability (Khalil et al., 2013). Additionally oral delivery of heparin is achieved by addition of GA (Motlekar et al., 2006).

To investigate the effect of GA as an absorption enhancer, F1 and F2 were given to rats with GA, added just prior to administration or were included in the organic phase during AmB loaded to PLGA-PEG NP preparation. AmB mean plasma concentration-time profile after single oral doses of AmB-loaded PLGA-PEG formulations with and without the addition of the absorption enhancer GA are depicted in Figure 3.6 and the pharmacokinetic parameters are presented in Table 3.9.

C_{max} after F1 without GA was 1.6 times that after Fungizone ($P<0.05$). A significant increase ($P<0.05$) in AmB C_{max} (2.3 fold) with 4.3 fold increase in AUC (332.3%) were observed after addition of 1% of GA to the PO formulations, just prior to administration. This is an indication of the efficiency of GA as an absorption enhancer for AmB. The

Increasing the GA content from 1% (F2-GA-out-1) to 2 % (F2-GA-out-2), showed a significant increase ($P <0.05$) in C_{max} (2.97 fold) and in AUC (390.7%) compared to F1 formulation without GA. Further increase in GA content to 3% showed no significant change in either C_{max} or AUC of AmB profile, data not shown. Therefore, GA at 2% was selected as the optimum amount to be added to the rest of tested formulations.

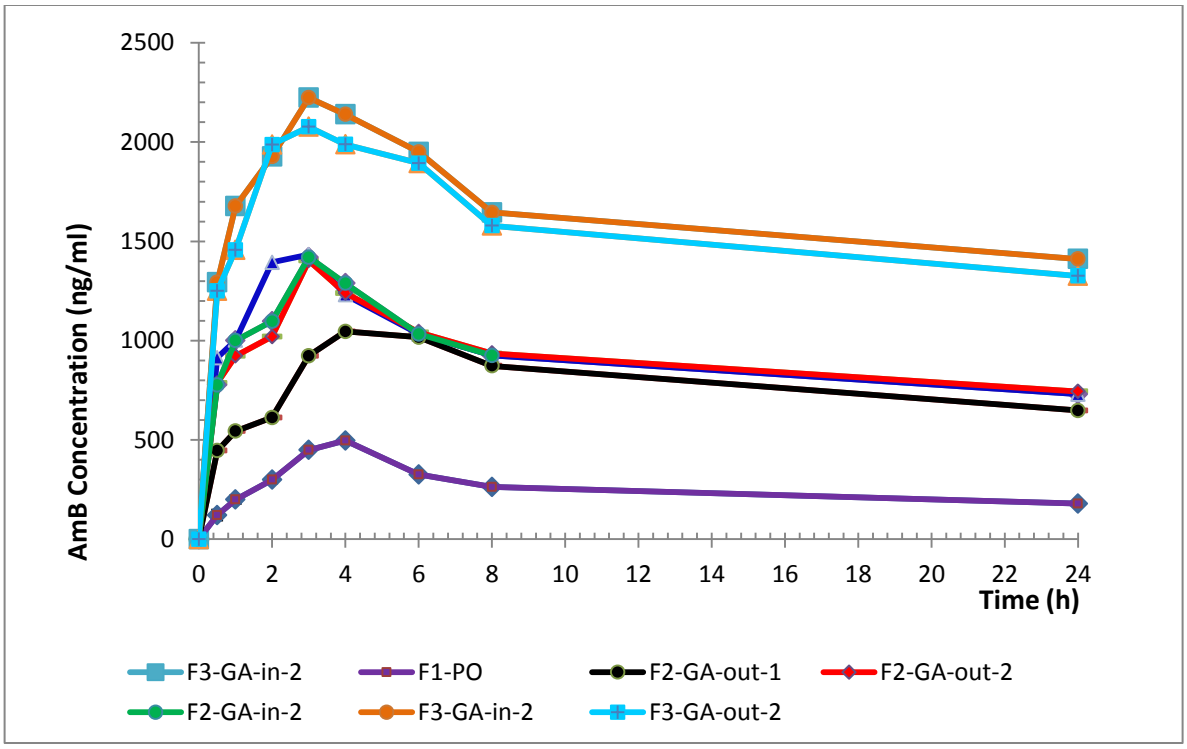


Figure 3.6. Mean plasma AmB concentration-time profiles (mean ± SD) following single oraladministration of 10.0 mg/kg of AmB-loaded PLGA-PEG formulations in rats (n=6).

Table 3.9. Pharmacokinetic parameters of Amphotericin B after a 10 mg/kg siingle oral administration of AMB as Fungizone® and AMB-Loaded PLGA-PEG NP formulations in rats (n=6).

Parameters	F1-PO	F1-GA-out-1	F1-GA-out-2	F1-GA-in-2	F2-GA-in-2	F2-GA-out-2	Fungizone®-PO
AUC (µg.h/l)	12325.1 ±1511	53287.0 ± 24997	60478.8 ± 7437	88307.9 ±36092	120689.8 ± 30628	108366.9 ± 19736	9836.3 ± 1654
C_{max} (ng/ml)	480.2 ± 38.7	1115.0 ± 172	1426.3 ± 133	1239.4 ± 75	2387.2 ± 294	2147.2 ± 176	298.2 ± 28
k_a, /h	0.994± 0.196	0.72±0.35	1.244± 0.513	1.684±1.374	1.68±1.15	1.35±0.38	0.904±0.0.72
$t_{1/2}$ (h)	24.0 ± 4.4	34.6 ± 15.1	36.0 ± 5.5	42.7 ± 4.2	41.3 ± 11.5	37.1 ± 6.5	35.3 ± 2.6
Mean F	0.015	0.063	0.072	0.105	0.072	0.064	0.117
Mean $F_{rel, F1}$	-	4.3	4.9	7.2	4.9	4.4	0.8
Mean $F_{rel, Fungizone}$	1.3	5.4	6.1	9.0	6.1	5.5	-

It was noticed that the addition of GA during preparation process (F2-GA-in) or just prior to administration (F2-GA-out) showed no significant difference ($P > 0.05$) in the AUC(13.5%), with a 27.9% increase in the C_{max} value. This is an indication that the efficacy of GA as an absorption enhancer for AmB is not changed either GA added just prior to administration or during the preparation.

Increasing the amount of the AmB inside the formulation from 20 to 40 mg (F3-GA-in-2 and F3-GA-out-2) did not showed any significant increase in the AUC after amount normalization comparing to F2 formulations. Therefore, within the tested amount in the formulation, AmB shows linear kinetic.

It should be mentioned that for each tested formulation the value of ka was much greater than the value of k which indicates a fast release of AmB with no sign of the flip-flop' model (Gibaldi and Perrier, 1982). The highest ka among the tested formulations was F1-GA-in compare to the others. Therefore, the addition of GA during preparation of F1 resulted in higher release of AmB.

The absolute bioavailability (F) of AmB from tested AmB loaded to PLGA-PEG NP formulation was calculated, Table 3.10. Upon the addition of 2% GA during the formulations, the F was improved from 1.5 to 10.5% (600 folds). The F_{rel} was improved from 332 to 616.5% and 25 to 800% after the addition of GA compared to F1-PO and Fungizone®, respectively. The highest improvement in F_{rel} was detected in formulation F1-GA-in-2.

Table 3.10. Bioavailability of AmB in rats after Fungizone® PO administration in comparison to different novel AmB-loaded NPs administered intravenously and orally in different doses

Reference	Formulation /Polymer	Dose of Fungizone, mg/kg	AmB Dose in tested Formulation, mg/kg	F_{rel} to Fungizone %
Current study	Copolymer (PLGA-PEG)	10	10	798
(Yang et al., 2012)	Cubosomes	10	10	285
		10	20	702
(Italia et al., 2009)	PLGA	10	10	693
(Sachs-Barrable et al., 2008)	Peceol/AmB	50	50	2197
		50	5	8506

3.11. Conclusion

A simple, rapid and sensitive analytical assay was developed and validated for the analysis of AmB in rat's plasma. One of the advantages of this method is the use of a plasma protein precipitation procedure for sample preparation, which is less expensive than solid phase extraction, less time consuming and more environmentally friendly than liquid extraction since a lot of organic solvent has to be evaporated. Moreover, the method requires considerably less plasma, which is important for pharmacokinetic studies involving repeated blood sampling. The chromatographic runtime is also short. Therefore, the developed bioanalytical method can be reliably employed as an assay method for pharmacokinetic study of any dosage form containing AmB in rats or human.

In conclusion, employing PEGylated Diblock copolymer (PLGA-PEG) is crucial for improving the oral absorption of AmB over the presence of sodium deoxycholate as in Fungizone. These polymeric NPs has the ability to cross the mucosal barrier by potential endocytic uptake mechanisms thus could enhance lymphatic transport of AmB in the particulate form. In the meantime, the addition of GA 2% to the AmB loaded to PLGA-PEG shows a tremendous improvement of AmB oral absorption by > 790 % than Fungizone. It should be notice that there is no significant difference when adding the GA inside "during" preparation or outside "just prior" to PO administration.

The amount of AmB could be increased up to 40 mg per patch without affecting the stability of the drug. Therefore, in this study an innovative AmB oral formulation was developed which would improve the patient adherence with potential reduction of adverse effect due to the only route (iv) AmB administration.

Limitation of this study, this study was a single-dose study in a small number of rats. A repeated dose pharmacokinetic study, to maintained AmB levels at steady state, is required to design the best dosage regimen for patients. Although individual variation was typical for pharmacokinetic studies, the small sample size is certainly an important however; statistical significance was reached in many of the pharmacokinetic endpoints for the iv vs. PO comparisons.

Chapter IV. *In Vitro* and *In Vivo* Cytotoxicity Monitoring of Amphotericin- B Loaded to PLGA-PEG Copolymer

4.1. Introduction

Fungal infections due to the fungi *Candida* and *Aspergillus* are associated with morbidity and mortality in immune compromised individuals and prolonged hospitalization.

Rapid treatment with AmB or fluconazole is required to reduce the death rate observed in these patients(Espuelas et al., 2003).Severe side effects associated with the administration of its water soluble intravenous (iv) formulation, Fungizone®, such as nephrotoxicity, have restricted the clinical uses of AmB. AmB-associated nephrotoxicity can be classified as glomerular or tubular. The clinical and laboratory manifestations of glomerular toxicity include decreases in renal blood flow and glomerular filtration rate. Tubular toxicity is manifested by the presence of urinary casts, hypokalemia hypomagnesemia, renal tubular acidosis and nephrocalcinosis (Torrado et al., 2008, Chuealee et al., 2011, Burgess and Birchall, 1972).

The anti-fungal activity and anti-leishmanial effect of AmB is correlate to an ion channel model, where AmB forms a stable ion channel in the presence of ergosterol found in the fungal cell membrane (De Kruijff and Demel, 1974, Gruszecki et al., 2003, Yamamoto et al., 2015). The higher selectivity for ergosterol over the mammal counterpart, cholesterol, is thought to account for its pharmacological activity. Recently, another mechanism called the 'AmB sponge model' has also been proposed, in which AmB is thought to extract ergosterol from the fungal membrane to form extra-membranous aggregates (Anderson et al., 2014, Yamamoto et al., 2015).

AmB toxicity is mainly associated with its binding to the phospholipid bilayer, specifically ergosterol and cholesterol, present in fungal and mammalian cells respectively. AmB interacts with these components of the cell membrane and forms ion channels. These ion channels disrupt membrane functions increasing cell membrane permeability leading to loss of cell viability by leakage, first of ions, then other cellular contents. A schematic presentation of the interaction between AmB and phospholipid bilayers is shown in Figure 4.1. The selective toxicity of AmB is mainly due to its greater affinity towards ergosterol forming larger channel pore sizes than in cholesterol-containing membranes in mammalian cells (Brajtburg and Bolard, 1996, Matsuoka and Inoue, 2009).

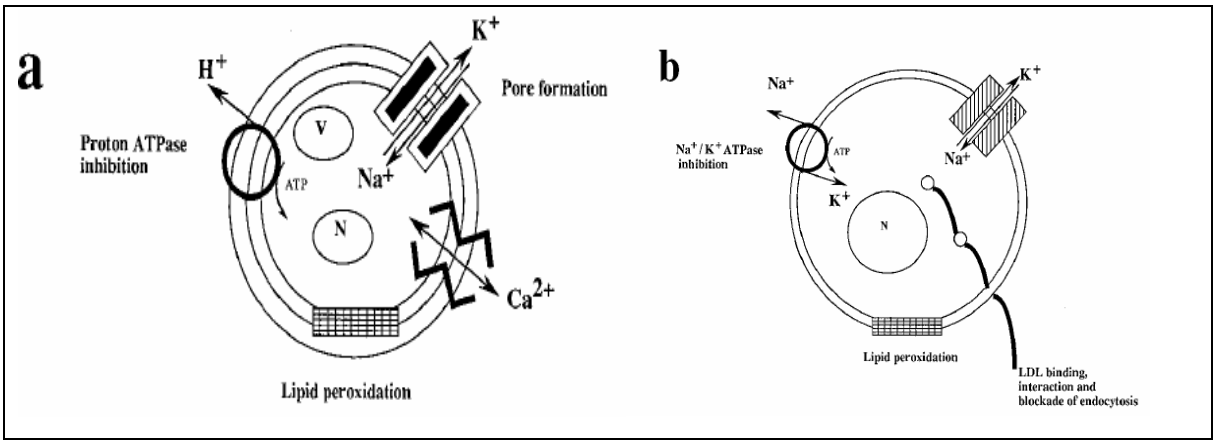


Figure 4.1. Possible sites of Amphotericin atack against fungal cells (a) and mammalian cells (b)(Brajtburg and Bolard, 1996).

AmB state (monomers or aggregates) affect its efficacy and toxicity, since monomers form are responsible for the therapeutic activity, on the other hand, the aggregates forms were responsible for drug toxicity(Nishi et al., 2007). AmB interact with the sterols present in the membranes in different ways. In fact, AmB induces leakage of intracellular potassium through formation of self-associated AmB (oligomers) in the mammalian cholesterol containing membranes and toxicity in fungal cells related to the monomeric form of AmB binding to ergosterol(Brajtburg and Bolard, 1996).

AmB deoxycholate (Fungizone®) related toxicity might be classified as acute or chronic. Acute or infusion-related toxicity is characterized by fever, chills, rigors, malaise, generalized aches, nausea, vomiting, and headache. Hypertension, hypotension, hypothermia and bradycardia are other reported infusion-related toxic effects of administration of AmB deoxycholate.

Nephrotoxicity is the most serious chronic adverse effect of AmB; the serum creatinine concentration increases in more than 80% of patients receiving this drug(Sabra and Branch, 1990, Tonomura et al., 2009). Nephrotoxicity is defined in most studies as a doubling of baseline creatinine (Cr) levels (100% increase from the serum baseline) or greater than 2.5 mg/dl (Miller et al., 2004). The incidence of AmB nephrotoxicity is very high, varying in studies between 49% and 65%(Deray, 2002).

In the conventional solution formulation, the drug is present as micelles. As soon as the drug solution enters the bloodstream it separates from the micelles and leads to the toxic effects. The Development of three new lipid-based formulations in 1990that are clinically used(AmBisome®,Amphocil®, and Abelcet®)on the other hand, release AmB slowly, hence are less toxic (Clements Jr and Peacock Jr, 1990, Baginski et al., 2005, Torrado et al., 2008).It is retained in the monomer state (active form) and is incapable to form the aggregates state (toxic form), resulting in significant increase in AmB activity with lower toxicity. (Espuelas et al., 2003). AmB lipid formulation are available as parenteral therapy. However, unpredictable pharmacokinetics, toxic effects at higher AmB doses, infusion-related reactions and high costs, have limited the benefit of these formulations in clinical settings(Falamarzian and Lavasanifar, 2010a).

Additional studies have shown that decrease in AmB toxicity was related to a drop in AmB anti-fungal activity (Espuelas et al., 2003). Several studies have shown that higher doses of new lipid formulation are needed to attain an equivalent effect of a small dose of Fungizone® for fungal infection treatment.

Alternative to AmB-lipid formulations, the nanostructure-based formulations, such polymeric NPs, have demonstrated in several literatures antifungal efficacy and lower toxicity, decrease in AmB dose and increase in the accessibility of the drug to organs and targeted tissue(Amaral et al., 2009, Laniado-Laborín and Cabrales-Vargas, 2009, Souza et al., 2015). Other researchers report that certain carrier vehicles added during the formulation process affect the aggregation state and activity of AmB (Legrand et al., 1992). Barwicz etal. demonstrated the ability of surfactants to reduce the toxicity of AmB via decreasing the aggregation state of the drug (Barwicz et al., 1992, Adams and Kwon, 2003).

Most new AmB drug delivery systems have been examined in the context of their use as antifungals. Those preparations that have been tested against *Leishmania* have shown variable results though none has proved more effective than the commercial formulation. The ideal delivery system would solubilize AmB, have low cytotoxicity and heamolytic activity towards cells, actively target macrophages, achieve co-localization with the Leishmania parasite and have high anti-leishmanial activity(Brajtburg and Bolard, 1996).

Polymeric NPs to be a drug carrier, must be biocompatible and biodegradable, showing no toxic effects *in vitro* or *in vivo*(Gaucher et al., 2010, Kumari et al., 2010, Vilar et al., 2012). Moreover, PLGA-b-PEG copolymer, is able to form NPs easily, with well controllable size and good encapsulation efficiency (Locatelli and Franchini, 2012).

It was hypothesized that conjugation of AmB to stealth PLGA-PEG copolymer will solubilized the drug and decreases the toxicity through slow release of the drug. The conjugation of the drug with PLGA-PEG copolymer might thus prevent its aggregation and thereby decrease its toxicity toward mammalian cells, while maintaining it in a monomeric form that favors antifungal activity (Brajtburg and Bolard, 1996, Torrado et al., 2008). Furthermore, it was further hypothesized that freeze dried formulations of AmB loaded PLGA-PEG could be reconstituted in water and retain therapeutic activity.

In previous Chapters, non-lipid formulations of AmB utilizing nanotechnology through AmB loaded to PLGA-PEG copolymer were developed and characterized. Both *in vitro* and *in vivo* studies have demonstrated good results, increased solubility, improved bioavailability and higher drug loading was observed. This Chapter will examine in more detail the toxicity of selected novel oral AmB –NPs formulations in *in vitro* and *in vivo* studies.

Toxicity has been evaluated by several tests; *in vitro* measuring of the hemolysis, *in vitro* measuring the antifungal activity, *in vitro* analysis of the cell viability by MTT assay, and lastly by *in vivo* nephrotoxicity and histopathological evaluation were conducted in rats kidneys and liver. Clinical chemistry parameters, such as blood urea nitrogen (BUN) and plasma creatinine (PCr) were also evaluated to determine the degree of toxicity of new developed AmB formulations.

4.2. Materials and Apparatus

4.2.1. Materials

RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin and streptomycin were purchased from Gibco-BRL. Sabouraud Dextrose Agar (SDA) plates (RODAC™) obtained from BD Diagnostics.

All other chemicals were of analytical reagent grade, and solvents were of HPLC grade and were obtained from local commercial suppliers.

4.3. Methods

Novel oral formulations of AmB NPs were prepared by an emulsification diffusion method as previously described using stealth AmB loaded PLGA-PEG copolymer see Chapter II for more details. The drug amount in each formulation was 20 mg with the exception of C7 formulations which contained 40 mg of AmB. The selected formulations listed in Table 4.1, were used in *in vitro* and *in vivo* toxicity investigations.

Table 4.1. The composition of the selected formulations used in toxicity studies

Formulation	Carrier/ Polymer
Fungizone®	Colloidal dispersion of AmB and sodium deoxycholate
A6	Diblock, PLGA–PEG 6000; Lactic to glycolic acid ratio (1:1) with 10%PEG , 5000 Dalton molecular weight(RGPd 50105)
B6	Triblock, PLGA–PEG 6000-PLGA; Lactic to glycolic acid ratio (1:1) with 10% PEG , 6000 Dalton molecular weight (RGPt 50106)
C6	Diblock, PLGA-PEG (Lactic to glycolicacid ratio1:1 molar ratio)with 15% PEG; molecular weight 6000 Dalton RGPd 50155
C7	Diblock, PLGA-PEG (Lactic to glycolicacid ratio1:1 molar ratio)with 15% PEG; molecular weight 6000 Dalton RGPd 50155
D6	Diblock PLGA–PEG 6000; Lactic to glycolic acid ratio (1:1) with 5% PEG , 5000 Dalton molecular weight RGPd 5055

4.3.1. In vitro Assessment the Hemolytic Activity of AmB

In vitro hemolytic activity of AmB-loaded PLGA-PEG copolymer formulations were assessed using isolated rat red blood cells (RBCs) according to Jain and Kumar (Jain and Kumar, 2010). Briefly, blood from healthy Sprague-Dawley male rats (250-350 g) was collected by cardiac puncture (with technician aid from research center) under anesthesia directly in to heparinized blood collecting vials. The red blood cells (RBCs) were separated by centrifuging the whole blood at 3000 rpm for 15 minutes, and then discarded the supernatant. Thrice washed of the RBCs with isotonic phosphate buffer saline (PBS- 0.15 M) at pH 7.4. Then RBCs was dispersed in PBS to obtain 1% hematocrit. RBCs were used on the same day for further experiments. Subsequently, 1.0 ml of the RBCs suspension was

mixed in 1.0 ml of PBS containing 20, 50 and 100 µg/ml AmB equivalent formulation (Fungizone® and selected formulations of freeze-dried AmB-NPs;A6, B6, C6, C7 and D6) and incubated at 37^0C in a shaking water bath at 100 rpm. The experiment was performed in triplicate. After 8 and 24 hours of incubation (Serrano et al., 2015), haemolysis was stopped by reducing the temperature to 0^0 C and unlysed RBCs were removed by centrifugation for 10min at 3000 rpm. The supernatant was collected and the erythrocyte pellet was lysed with sterile distilled water then analyzed for the extent of haemolysis using a spectrophotometer set to 540 nm (the absorption maximum of haemoglobin). Control RBC (2×10^8 cells per ml) incubated with PBS alone were used to estimate the total haemoglobin content. Results were expressed as a percentage of hemolysis as given in the following equation:

$$\% \text{ Hemolysis} = [(Abs_S - Abs_0) / (Abs_{100} - Abs_0)] \times 100$$

Where Abs_S is the absorbance of the sample, Abs_0 is the average absorbance of the buffer; negative control, and Abs_{100} is the average absorbance of the lysed samples (in purified water; positive control).The remaining haemoglobin was calculated as a percentage of the total content. Results are given as the mean of one experiment representative of three experiments carried out with each concentration in triplicate.

4.3.2. In vitro Antifungal Activity

One yeast strains *C. albicans* was used for susceptibility testing. The minimum inhibitory concentrations (MICs) of AmB-loaded PLGA-PEG copolymer formulations (Fungizone® and selected formulations of freeze-dried AmB-NPs; A6, B6, C6, C7 and D6) were determined by broth dilution according to the National Committee for Clinical Laboratory Standard "NCCLS document M27-A,(Standards, 2002). Briefly, *Candida albicans* (ATCC 90028) cell suspensions of ~1x 10^6 cells/ml were diluted 1:50 in RPMI-1640 growth medium and 100 µl dispensed into a microliter tray containing a serial concentration of AmB 0.05-1.5 µg/ml. A solution of 5 mg/mL was prepared in DMSO for free AmB and in water for the selected created formulations AmB-NPs copolymer immediately before use.

The tray was then incubated for 24 and 48 hours at 37^0C. Yeast inoculation were cultures grown on Sabouraud Dextrose Agar (SDA) plates and inoculated into RPMI 1640 broth medium to yield a final inoculum concentration of 10^4 yeast cells/ml and checked by doing a viable colony count on SDA plates. Two wells containing drug-free medium and inoculum were used as controls. The inoculated plates were incubated at 35^0C for 24 hours. The growth in each well was then estimated visually. The MIC was defined as the lowest drug concentration (MIC) that resulted in complete inhibition of visible growth. The MIC was recorded to be the lowest concentrations of the AmB that prevented visible growth of *C. albicans* and expressed in µg/ml. The end point was determined as the concentration to produce optically clear wells (MIC-0).

4.3.3. In vitro Cell Viability and Cytotoxicity Assays

All three cell lines (AR42J-B13,Jurkat and HL60) were grown at 37 ^{0}C in an humidified air atmosphere containing 5% CO_2.

AR42J-B13 Cells: Rat pancreatic cells that differentiate to hepatocyte cells, by adding 10 nM dexamethasone, many of the cells underwent a phenotypic alteration to a form referred to in the present paper as B-13/H cells, over a period of two weeks (Marek et al., 2003).Cells were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 20 mM HEPES, and 5 mM $NaHCO_3$. Cells were routinely cultured in T75 flasks (corning) at a cell density between 3-9 $x10^5$ /ml until required for specific experiments.

Jurkat Cells; Human T cell lymphoblast. Jurkats are suspension cells routinely grown in Roswell Park Memorial Institute Medium: RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2mM glutamine and 10 U/ml of penicillin/streptomycin (all from sigma). Cells were routinely cultured in T75 flasks (corning) at a cell density between 3-9 $x10^5$ /ml until required for specific experiments.

HL60 Cell; Human caucasian promyelocyctic leukemia cell line. Cultures were maintain in T75 flasks (corning) at a cell density between 1-9 $x10^5$ /ml in RPMI 1640 with 2 mM glutamine and 10-20% FBS until required for specific experiments.

The MTT (3-(4,5dimethylthiazoly)-2,5-diphenyl-tetrazolium bromide) colorimetric assay, as developed by Mosmann 1983(Mosmann, 1983), and modified by Edmondson

1988(Edmondson et al., 1988), was employed to assess the cytotoxicity of the selected prepared novel oral AmB-loaded NPs (Fungizone® and selected formulations of freeze dried AmB-NPs; A6, B6, C6, C7 and D6) on the cell lines. Cells were seeded in 12 well plates at a density of 3 x 10^5 cells per well, suspended in 1.0 ml medium. After incubation for 24 hours, cultures were treated with 125, 250 and 500, ng/µl and 100 µl culture medium alone (control), or with standard AmB or AmB-loaded PLGA-PEG copolymer selected formulations and incubated for another 2, 4, 6 and 24 hours. The cells were then pelleted by centrifugation at 1000 rpm for 3.0 minutes and then supernatant discarded prior to re-suspension in 0.5ml fresh medium without FBS (0.5 ml). The cells were then incubated with MTT through addition of 500 µg/ml of MTT and incubation for a further 2 hours at 37^{o}C and 5% CO2 in air. 800 µl of isopropranolol was then added to each well and the absorbance at 570 nm determined after 10 minutes incubation using a 96 well plate reader. The numbers of viable cell in the treated well were compared to those in the untreated well and estimated as % viability.

4.3.4. In vivo Nephrotoxicity and Hepatotoxicity Study

Nephrotoxicity studies were performed on male Sprague Drawly rats weighing 200-300 g. The animals were housed under standard conditions of temperature (22 ±2 ^{0}C), light and dark cycle (12/12 hours). The animals were kept on standard pellet diet and water *ad libitum*. The animals were randomly divided into eight groups (n=3). Group-I was kept as control without any treatment; Group-II was treated with Fungizone® as a slow intravenous (iv) injection; Group-III received iv blank NP with no AmB; Group-IV received iv administration of A6; Group-V received iv administration of B6; Group-VI received iv administration of C6; Group-VII received iv administration of D6; Group-VIII received iv administration of C7. The iv Blood samples were withdrawn from the retro-orbital plexus and plasma was separated by centrifugation at 3,000 rpm for 15 min.

A single dose experiment consisted of a single dose of AmB or its equivalents dose as 1.0 mg/kg of body weight from each of the selected AmB-NP formulations (Fungizone® and selected formulations of freeze dried AmB-NPs; A6, B6, C6, C7 and D6). The 7-days repeated dose experiment consisted groups that were dosed with 1.0 mg/kg body weight of

AmB from each formulation. 24 hours post-dose, blood samples were withdrawn from the retro-orbital plexus and plasma was prepared by centrifugation at 3000 rpm for 15 min. Blood urea nitrogen (BUN) and plasma creatinine (PCr) were measured using an automatic analyzer 7180 (Hitachi High-Technologies Co., Tokyo, Japan). Alkaline phosphatase (ALP IU/L), γ-glutamyltransferase (γ-GT, IU/L), alanine aminotransferase (ALT. IU/L) were determined Automatic analyzer 7180 (Hitachi High-Technologies Co., Tokyo, Japan).

For histopathological analysis, one kidney from each rat was fixed in neutral buffered 10% formalin for ≥48 h, bisected and embedded in paraffin. Sections of 5.0 µm thickness were cut from each kidney, and stained with haematoxylin and eosin. For histopathological analysis of liver, a piece of median lobe from the livers were removed from each rats and placed immediately in 10% neutral buffered formalin. Sections of 5.0 µm thickness were cut and stained with haematoxylin and eosin similarly to kidney tissue.

4.3.5. Statistical Analysis

The Student t-test was used to examine the difference and one-way analysis of variance (ANOVA) was employed to evaluate the reproducibility of the assay. Other tests were used using IBM SPSS Statistics 21. A p-value < 0.05 was considered significant.

4.4. Results and Discussion

4.4.1. In vitro Hemolysis Test

AmB is it reported to be highly toxic in its aggregated state than in its monomer form (Brajtburg and Bolard, 1996, Nishi et al., 2007). In solution, AmB exists in three different forms; monomers, oligomers and aggregates. The soluble form of AmB exists in monomeric form (Brajtburg and Bolard, 1996, Nishi et al., 2007).

The extent of AmB aggregation can be measured by the ratio of absorbance at 348 nm to 409 nm (Legrand et al., 1992). Barwicz et al. (Barwicz et al., 1992) report the ratio to be 2 for aggregated species. Fungizone® has a value of 2.9 (Mullen et al., 1997). For AmB-loaded PLGA-PEG copolymer, the values obtained were less than 1.14 showing that AmB

was not aggregated in the novel oral formulation. Therefore, it is essential to measure the heamolytic toxicity for any new AmB formulation.

The degree of hemolysis observed after incubation of RBCs with selected formulations (A6, B6, C6, D6 and C7) of AmB-NPs in comparison with Fungizone® is depicted in Figure 4.2. After 8 h of incubation, the hemolysis rate was high (84%) with Fungizone® at 100 µg/ml and it was reduced to 43% at lower concentration of 20 µg/ml. Therefore, Fungizone® is likely to be toxic, even at the lowest concentration used in the experiment and it was higher than water since AmB lyses RBCs by active process of pore formation. It was also noticed that the hemolysis was dose dependent. At a similar concentration, AmB-loaded to the selected NPs formulations showed negligible hemolysis. Regarding the degree of hemolysis, the different tested formulations were classified as low hemolytic toxicity 4 to 8 times hemolysis reduction in comparison with Fungizone®, both C6 and C7 formulation showed the lowest hemolysis.

The lower hemolysis observed for the AmB-loaded PLGA-PEG copolymer, perhaps reflects a better control over the rate of AmB diffusion from these selective formulation over the Fungizone®, indicating that the release of AmB from the PLGA-PEG copolymer is slow.

The obtained hemolysis data for all AmB-loaded PLGA-PEG copolymer, after 24 hour incubation, showed similar results as after 8 hour incubation, indicating that the releases of AmB from these formulations is slow.

Fungizone® caused higher extent of hemolysis because Fungizone® formulation consist of micellar dispersion of AmB with sodium deoxycholate, which act as surfactant and can encourage hemolysis itself in addition to the hemolysis caused by AmB. Fungizone® is acknowledged for causing hemolysis mostly due to pore formation altering electrolyte balance in erythrocytes (Yu et al., 1998, Fukui et al., 2003b, Bang et al., 2008, Nahar et al., 2008, Italia et al., 2009, Falamarzian and Lavasanifar, 2010a, Jain and Kumar, 2010, Shao et al., 2010b, Sheikh et al., 2010, Asghari, 2011).

The lack of hemolysis activity may be reflect the release of monomeric AmB from AmB-loaded PLGA-PEG Diblock copolymer as opposed to Fungizone®, which release both aggregated and monomeric forms of the drug (Yu et al., 1998, Adams and Kwon, 2003, Brajtburg and Bolard, 1996). During formulation, to dissolve AmB in the polymer

phase, a very small aqueous solution for acidification was added for 2 N HCL, thus precluding self-association or aggregation.

The results obtained therefore, indicates the protective effect of the PLGA-PEG Diblock copolymer in preventing the RBCs lysis of the formulations.

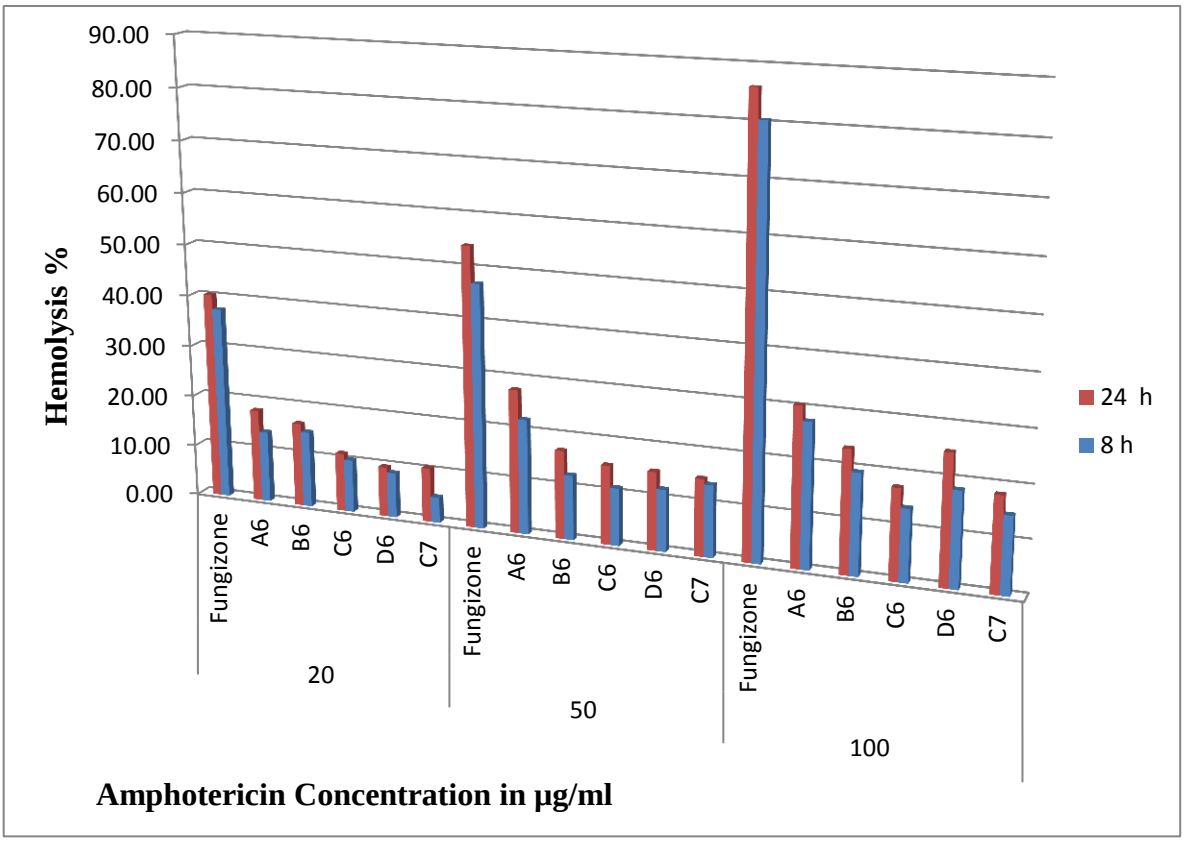


Figure 4.2.*In vitro* RBCs haemolysis following incubation of RBC with Fungizone® and different AmB-NPs formulation at concentrations of 20, 50 and 100 µg/ml. Values represent mean ± SD (n=3).

4.4.2. In vitro Antifungal Activity

Table 4.2. shows the antimicrobial activity of pure AmB, Fungizone® and three selected formulations of AmB-loaded to PLGA-PEG Diblock copolymers in *Candida albicans* after 24 and 48 h incubations. The MIC-0 for pure AmB was 0.5 µg/ml after 24 h and 1.0 µg/ml after 48 h incubation. Similar results were obtained by different researchers

(Nishi et al., 2007). Meanwhile, the MIC-0 for AmB-loaded to PLGA-PEG copolymer formulations was reduced ≥ 4-foldμg/ml.

AmB at concentrations > 0.5-1 fold the MIC had a fungicidal activity, while AmB at concentrations < 0.5-1 fold the MIC had a fungistatic activity, a finding that has been described previously by different groups of investigators (Tasset et al., 1992, Pfaller and Barry, 1994, Risovic et al., 2003, Belkherroubi-Sari et al., 2011). The MIC of AmB-loaded PLGA-PEG copolymer against *C. albicans* was reduced 2-3 fold compared with free AmB (Table4.2). This result indicates that developed AmB-NPs, have high therapeutic efficacy and are useful for the treatment of fungal infection including candidiasis.

Table 4.2. Checkboard assay of amphotericin B against *C. albicans*

	MIC-0 (μg/ml) after 24 h	MIC-0 (μg/ml) after 48 h
AmB	0.5 ± 0.01	1.0 ± 0.01
Fungizone®	0.5 ± 0.01	1.0 ± 0.01
A6	0.25 ± 0.01	0.25 ± 0.01
B6	0.25 ± 0.01	0.25 ± 0.01
C6	0.125 ± 0.01	0.125 ± 0.01
D6	0.25 ± 0.01	0.25 ± 0.01
C7	0.125 ± 0.01	0.125 ± 0.01

4.4.3. In vitro Cell Viability and Cytotoxicity Assays

The toxicities of AmB and the selected NPs formulations were tested in three cell lines (B-13/H, Jurkat and HL60) at different concentrations 125, 250 and 500 ng/ml tested after 2, 4, 6 and 24 h incubations. The Fungizone® was toxic in all tested cells (B-13/H, Jurkat and HL60) since the cell viability was < 80% (Figure 4.3- 4.5). These data showed variations in cell viability after testing different formulations, most of them were likely to be safe for the used cell lines, since in all cases > 80% of the three cell lines remained viable, even at 500 ng/ml. Moreover, these data showed a dose-dependent reduction in viability (with increasing the dose there is greater loss of viability). It can be concluded that

AmB-loaded to PLGA-PEG copolymer NPs of selected formulations were less toxic than the Fungizone®.

This observation is also consistent with what was obtained at 4, 6 and 24 h for the 3 cell lines (Figures4.4- 4.6). Another observation worthy of note is the effect of exposure time on cell viability. Increased cytotoxicity was observed with the B-13/H, Jurkat and HL60 cell lines as the duration of treatment increased from 4 h to 6 and 24 h.

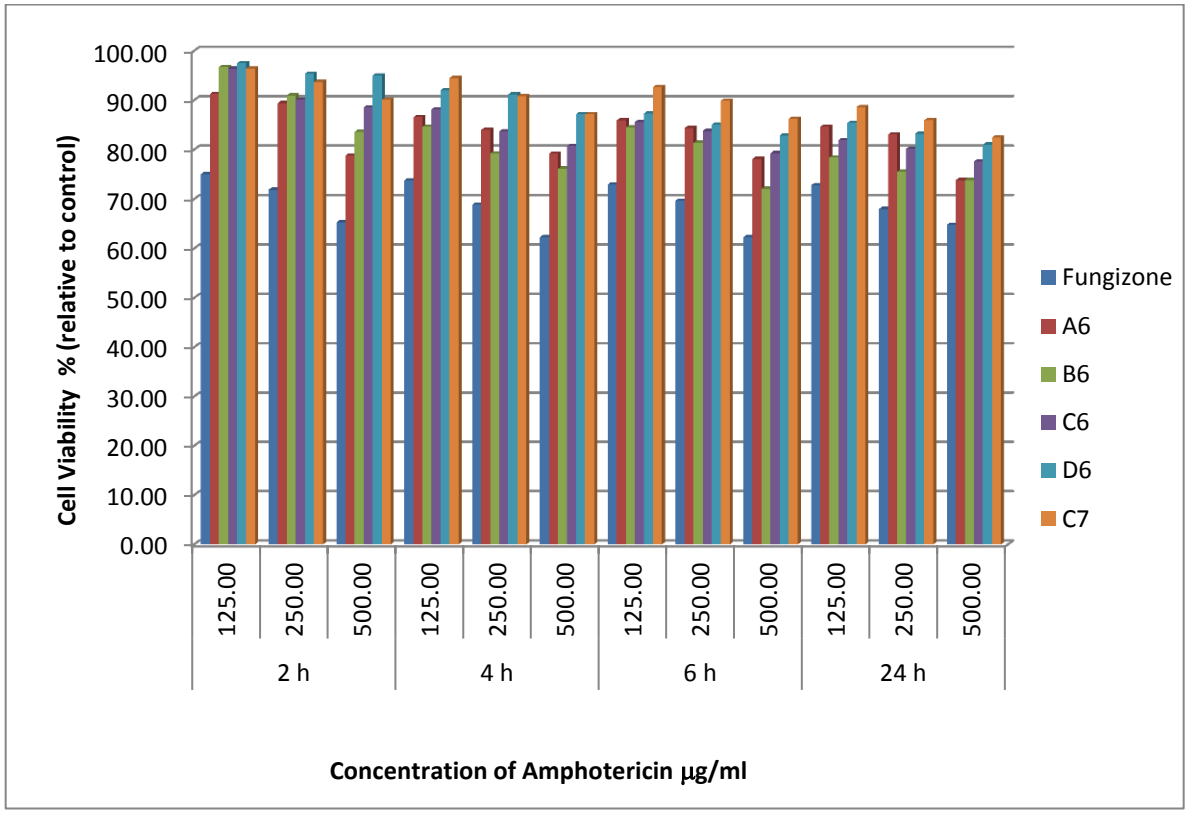


Figure 4.3. The viability of B-13/H cells after exposed to AmB (125, 250 , 500 ng/µl and control) in selected nanoparticles formulations after 2, 4 and 6 h determined by MTT assay (n=3)

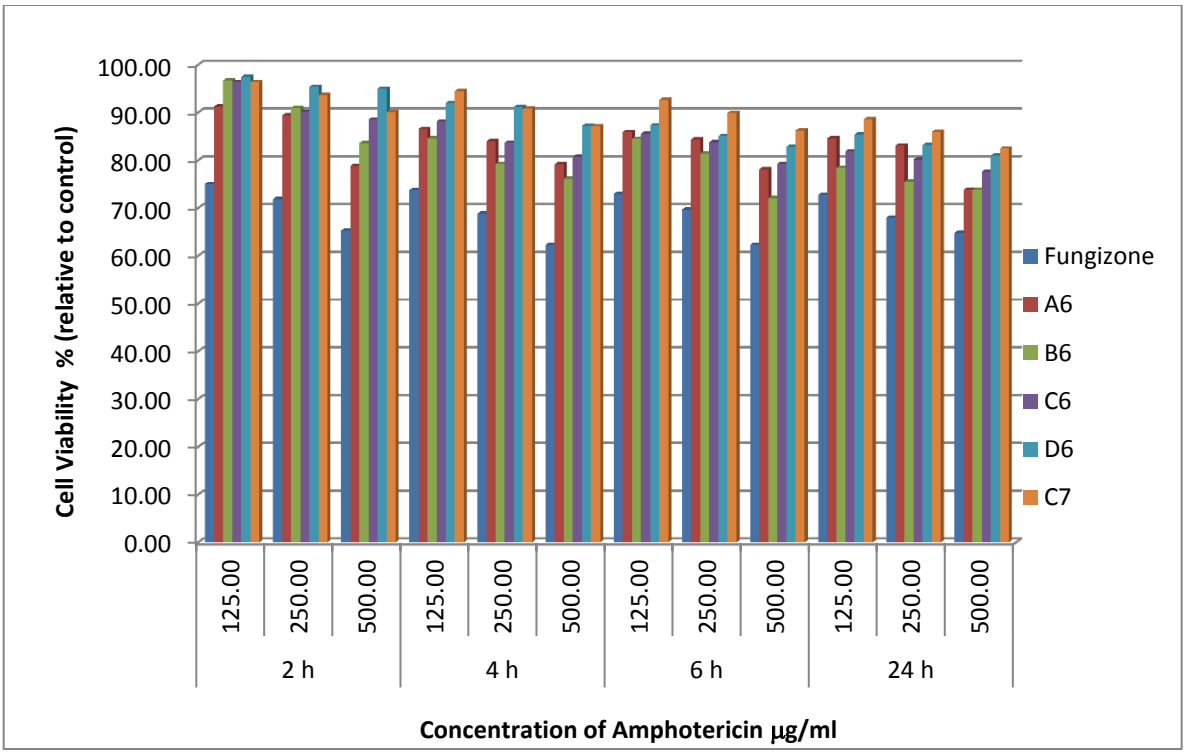


Figure 4.4. The viability of Jurkat cells after exposed to AmB (125, 250 , 500 ng/µl and control) in selected nanoparticles formulations after 2, 4 and 6 h determined by MTT assay (n=3)

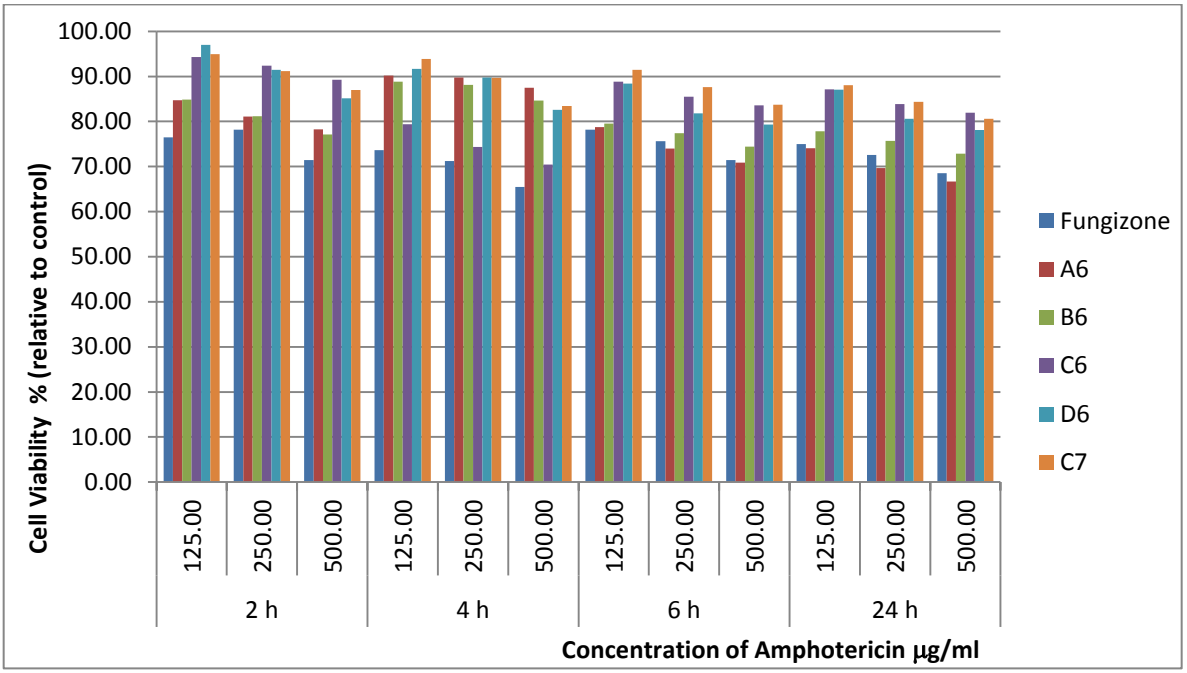


Figure 4.5. The viability of HL60 cells after exposed to AmB (125, 250 , 500 ng/µl and control) in selected nanoparticles formulations after 2, 4 and 6 h determined by MTT assay (n=3)

At higher AmB concentrations (above 250 and 500 µg/ml) there was no decrease in cell viability. In addition, with increasing the time, there was no significant ($P > 0.005$) differences in the cytotoxicity in all the three cell lines. Similarly, it was reported that AmB conjugated to carbon nanotubes had relatively small toxicity towards Jurkat cells, derived from a human T-cell leukemia (Wu et al., 2005). Furthermore, the effect of AmB conjugates on other cell types is still unclear. Although a cell line (such as Jurkat cells or HL60) is highly valuable to address questions regarding cytotoxicity, the results should be validated with primary cells such as hepatocyte cells. Studies with NPs of different chemical composition suggest that cancer cells are more resistant to NPs-mediated toxicity than primary cells found in the human body (Shakeri et al., 2015). Another study was conducted by Zahra and his coworkers (Um-i-Zahra and Zhu, 2015), on AmB-NPs cytotoxicity on three different hepatic cell lines (GRX, Hep G2 and ARL-6). They found that in an *in vitro setting,* AmB was able to selectively induce the cytotoxicity of GRX without affecting the liver parenchymal cells.

4.4.4. In Vivo Nephrotoxicity and Hepatotoxicity Study

AmB-induced nephrotoxicity is expressed by renal failure and abnormalities in tubular function. An elevated levels of both BUN and PCr are indicative of renal dysfunction or acute nephrotoxicity (Italia et al., 2009, Tonomura et al., 2009).

In this study, several functional and morphological renal changes were observed following single or multiple doses (for 7 days) of AmB or its selected NPs formulations. These changes were observed by analysis of blood chemistry and pathological examination. There was no mortality after iv dosing for a week of any of the rats during the study. Relevant changes in serum chemistry are summarized in Table 4.3. A significant increase in biochemical parameter's to test the kidney function (BUN, PCr) and liver function (ALP, γ-GT, ALT) after Fungizone® iv administration was observed in comparison to control group.

Table 4.3. Biochemical parameters after single and multiple i.v. dose administration of AmB and AmB-loaded PLGA-PEG copolymer as 1.0 mg/kg body weight to rats (n=3)

		Control	A6	B6	C6	D6	C7	Fungizone®
BUN (mg/dl)	SD	17.67 ± 3.05	41.67 ± 3.21	38.00 ± 3.51	20.0 ± 5.0	23.33 ± 4.16	18.67 ± 3.21	51.33 ± 2.0
	MD	18.67 ± 3.05	38.67 ± 4.62	36.00 ± 1.15	25.33 ± 3.05	31.00 ± 2.52	20.67 ± 2.08	50.67 ± 1.15
Cr (mg/dl)	SD	0.31 ± 0.03	0.57 ± 0.06	0.64 ± 0.03	0.35 ± 0.06	0.44 ± 0.06	0.34 ± 0.02	0.76 ± 0.02
	MD	0.28 ± 0.02	0.55 ± 0.06	0.59 ± 0.03	0.31 ± 0.03	0.41 ± 0.05	0.34 ± 0.03	0.73 ± 0.05
ALP (IU/l)	SD	457.00± 49.87	560.00 ± 42.03	553.00 ± 36.05	516.33± 48.13	559.00± 21.50	452.33± 26.63	583.00± 35.30
	MD	442.33± 30.43	549.33 ± 15.95	529.33 ± 35.13	521.00± 41.79	556.67± 15.52	456.67± 15.27	593.33± 25.17
γ-GT (IU/l)	SD	0.98± 0.22	0.96± 0.31	1.07± 0.27	0.81± 0.25	0.99± 0.23	0.89± 0.18	1.13± 0.17
	MD	0.78± 0.53	1.02± 0.22	1.17± 0.16	0.93± 0.25	0.99± 0.15	0.93± 0.18	1.12± 0.23
ALT (IU/l)	SD	29.66 ± 4.16	55.33± 6.03	44.33± 6.51	22.33± 6.24	24.33 ± 7.09	28.33 ± 10.15	45.00± 7.55
	MD	25.67± 4.18	57.33± 9.23	39.67± 5.69	25.33 ± 5.68	33.00 ± 6.56	31.00± 8.56	35.67± 8.33

SD: Single dose; MD: multiple doses

The main concern of this study was to determine the *in vivo* nephrotoxic potential of AmB in the selected NPs formulations. A substantial nephrotoxicity effect of Fungizone® was determined in rats with significant increase (P=0.003 and P=00.1) in BUN and PCr levels, respectively, as compared to the results in the control rats. In the meantime, a minimal nephrotoxicity (P<0.05) was observed with lower BUN and PCr levels in the rats, after iv administration the selected NPs formulations as compared to the Fungizone® (Table 4.3). The observed lower toxicity of AmB-NPs could be attributed to the slow release of AmB from the NPs formulations compared to its release from the deoxycholate micelles in Fungizone®. AmB is known to induce vasoconstriction, which leads to a

decrease of blood flow in the kidney. The observed reduction in the PCr is believed to be caused by a decrease in blood pressure that is associated with a decrease in blood flow in the glomerulus (Tasset et al., 1992, Tiyaboonchai et al., 2001, Risovic et al., 2003, Nahar et al., 2008, Italia et al., 2009, Tonomura et al., 2009, Belkherroubi-Sari et al., 2011).

The present study was further focused on the significance of the biomarkers, BUN and PCr for their sensitive detection of renal injury induced by AmB in rats. The rats measured BUN after the administrations of NPs formulations C6, D6 and C7 were within the normal level for rat plasma in these treatment groups, while after Fungizone®, A6 and B6, the mean BUN concentrations were > 40 mg/dl which consistent with moderate renal toxicity.

After C6 and C7 administrations the PCr values in rats were around 0.31 mg/ dl ,which is within the normal range for rat plasma. However, for the other treatment groups as shown in Table 4.3, the mean PCr was within the moderate renal toxicity (>0.4 mg/dl).

The histopathological analysis of kidney tissue after iv administrations of the NPs formulations of AmB and Fungizone®, did not confirm any unusual sign of necrosis in all treated groups except in the Fungizone® treated group. It showed a distinctive necrosis of various degree as presented in Figure 4.6, which has been approved by the PCr results in the same study.

Similar results were concluded with histopathology examination of liver tissues. It did not show any abnormalities in all treatment groups, except the Fungizone® one, which has clear necrotic hepatocytes and bile duct damage as indicated in Figure4.7.

As a conclusion, all the iv administrations of the developed AmB-loaded to PLGA-PEG Diblock copolymer investigated, showed minimal renal damage compared to the reference formulation Fungizone®, over a short duration of treatment.

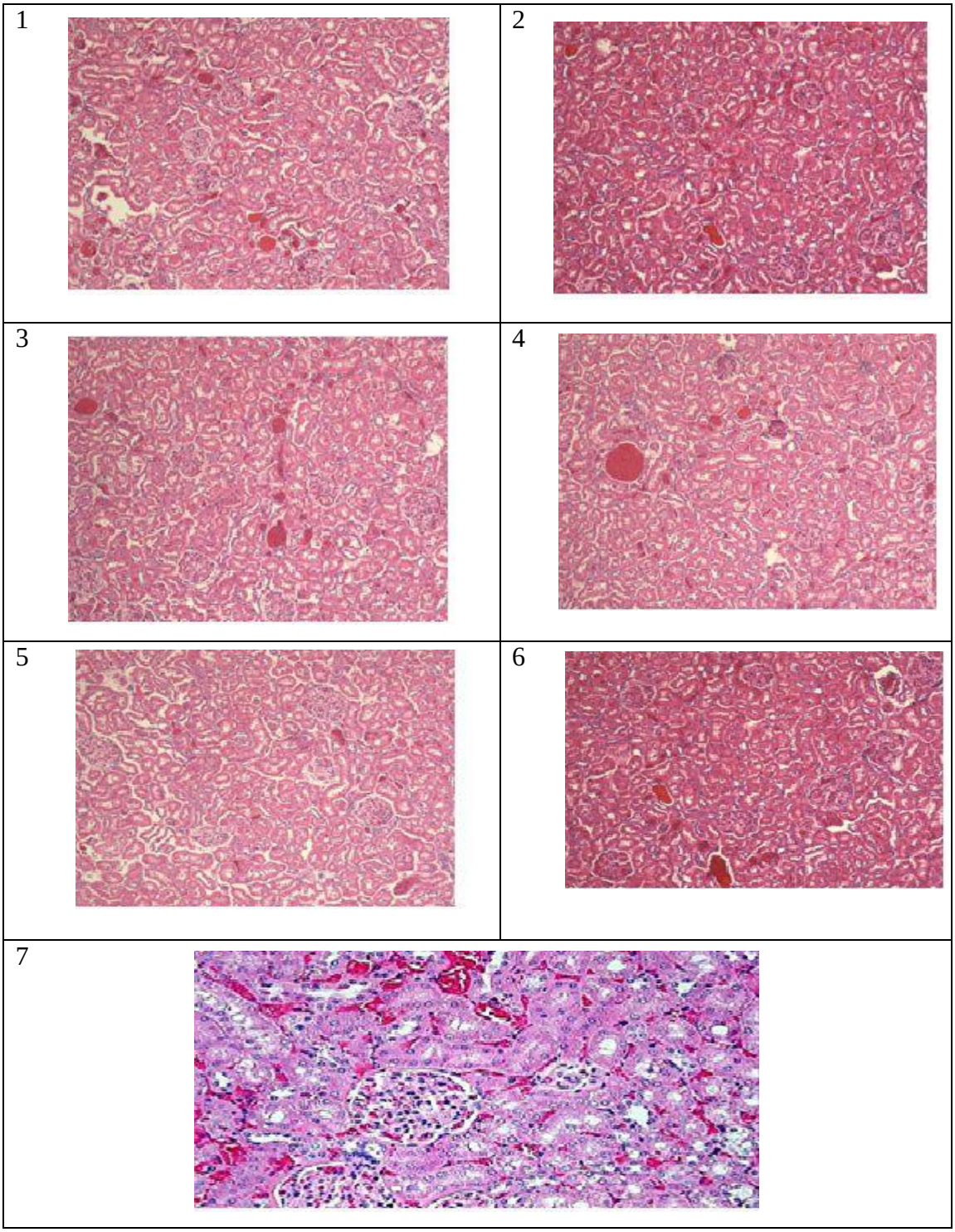


Figure 4.6. Typical kidney tissue alterations verified in rats treated with AmB or its equivalents dose as 1.0 mg/kg of body weight as iv administration of different AmB-PLGA-PEG copolymer. 1) Normal kidney tissue; 2,3,4,5,6 and 7) Varying degree of nephrotoxicity necrosis related to iv administration of as A6, B6, C6, D6, C7 and Fungizone® respectively.

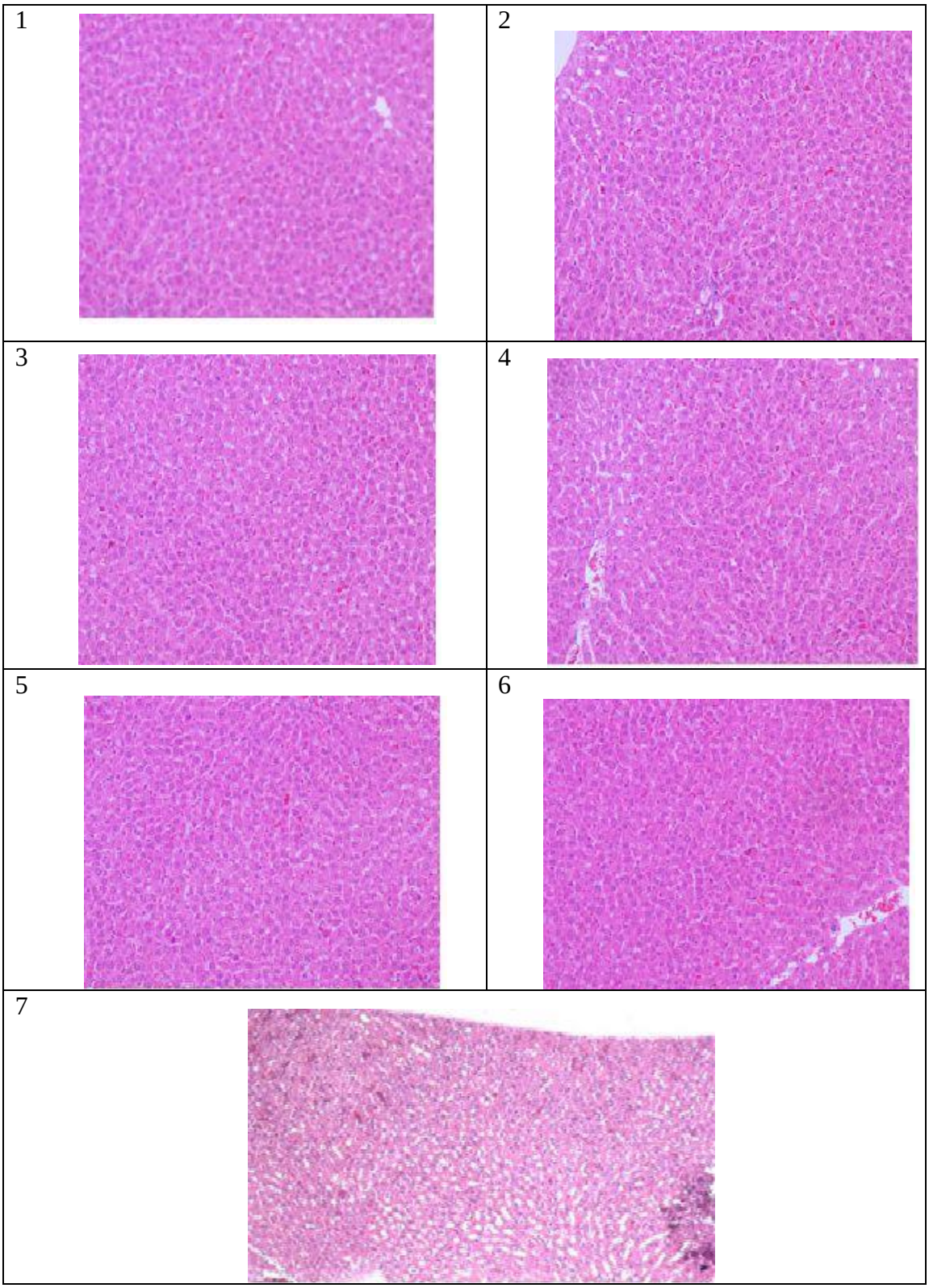


Figure 4.7. Typical liver tissue alterations verified in rats treated with AmB or its equivalents dose as 1.0 mg/kg of body weight as iv administration of different AmB-PLGA-PEG copolymer. 1) Normal liver tissue; 2,3,4,5,6 and 7) Varying degree of hepatocellular vacuolation related to iv administration of A6, B6, C6, D 6, C7 and Fungizone® respectively.

4.5. Conclusion

Novel oral biodegradable stealth polymeric nanoparticles have been successfully fabricated using the commercially available PLGA-PEG copolymer and loaded with AmB by an emulsification diffusion method. Drug loaded to PLGA-PEG has been characterized *in vitro* previously. Drug content and release studies demonstrated that A6, B6, C6, D6 and C7 formulations had the best drug release characteristics and higher drug content.

The freeze dried powder of the selected A6, B6, C6, D6 and C7 formulations produce milky colloid solution immediately after reconstitution in water. They drastically reduce the *in vitro* hemolytic activity of AmB as compared to Fungizone®. The lack of hemolysis activity may reflect the slow release of the drug from its PLGA-PEG Diblock copolymer. On a cellular level, AmB loaded to PLGA-PEG Diblock copolymer showed a potent activity against *in vitro C. albicans* in parallel with decreased cytotoxicity against three *in vitro* tested cell lines. Additionally, no pathological abnormalities were observed in rats given iv administrations of AmB NPs formulations in contrast to Fungizone® administration.

AmB induced nephrotoxicity could be detected by BUN and PCr measuring after administration in rats. A significant nephrotoxicity was detected after iv Fungizone®. The *in vitro* and *in vivo* studies of the selected AmB NPs formulations showed a minimal occurrence of the undesirable side effects caused by this drug, especially nephrotoxicity. Additionally, NPs did not induce hemolysis. Together all these results confirm the novelty of the developed oral NPs delivery system of AmB-loaded to PLGA-PEG Diblock copolymer formulations which could be a promising oral dosage form of AmB for the treatment of fungal infections. Formulations C6 and C7 were the best among the other tested ones.

Chapter V. General Discussion

In this study a innovative oral delivery of AmB have been developed, with solubility and bioavailability improved associated with lower toxicity. The optimization of the oral formulations have been passed through many stages, including; selection of the copolymer used PEGylated polymer, which consist of PLGA-PEG, having two parts a hydrophobic polyester part (PLGA) and a hydrophilic (PEG) part. Both parts have been approved by FDA for oral and parenteral administration in humans, additionally, both of these parts have been improved the solubility and bioavailability of different groups of drugs when used individually.

AmB loaded to PEGlyated polymer (PLGA-PEG) were successfully fabricated using the emulsion diffusion method, this method was efficient and reproducible with considerable efficient drug content.

Other factors were affecting the nanosized of the produced AmB-loaded NPs such as the stirring rate, cosolvency, pH, the addition and concentrations of stabilizers ans/or surfactant used.

In vitro characterizations of the AmB loaded to PLGA-PEG formulations showed the highest main particles size (MPS) of 1,068.1 ± 489.8 nm corresponding to A3 (RGPd 50105) and the lowest MPS 23.8 ± 4.8 nm corresponding to C6 (RGPd 50155). The decreasing in the MPS is mainly related to increasing the stirring rate from 8000 rpm to 24000 rpm and due to the addition of stabilizer PVP, miglyol and Vitamin E (TPGS). All these factors were contributed to the significant reduction in the MPS. The best polymer used during the study was C (RGPd 50155) consisting from diblock PLGA-PEG copolymer with 15% PEG in comparison to other used polymers A (RGPd 50105) consisting of diblock PLGA-PEG copolymer with 10% PEG, polymers B (Rgp t 50106) consisting of triblock PLGA-PEG copolymer with 10% PEG, polymers D (RGP d 5055) consisting of diblock PLGA-PEG copolymer with 5% PEG, and lastly polymers E (R 203 H) consisting of monoblock poly(D,L-lactide).The reduction of the MPS of the novel oral AmB loaded PLGA-PEG was associated with increment of drug entrapment efficiency (DEE). The maximum drug entrapment efficiency of AmB was increased up to 56.7%, with 92.7% drug yield.

Other physicochemical studies were evaluated also for the newly formulated AmB loaded to PLGA-PEG such as scanning electron microscope (SEM) and transmission electron microscope (TEM). The SEM and TEM images indicate isolated and non-agglomerated nanometric circular particles. Furthermore the Fourier infrared spectroscopy (FTIR) changes obtained from different AmB loaded to PLGA-PEG NPs formulations suggesting some sort of interaction between the drug and the polymer used.

A new simple, fast, accurate and reliable HPLC method for the *in vitro* investigations (drug content and drug release from the formulated NPs) of AmB has been developed. It is involving of reverse phase high performance liquid chromatography using acetonitrile as main constituent of the mobile phase and protein precipitation method and Nicardipine used as internal standard. A linear relationship has been obtained with the absorbance of the AmB and the concentrations used which in the range of 250-5000 μg/ml.The intra-day and inter-day variability were ≤ 6.2% with excellent linearity (R^2=0.9982) and reproducibility, short run time, with the average retention times of 2.87 ± 0.05 for the AmB 4.20 ± 0.09 min for the Nicardipine (IS), respectively with no interfering peaks. Thus, this HPLC method was reliable for determination of the AmB concentration and has been used for drug content calculation and the percentage drug yield as well as *in vitro* drug dissolution studies.

The main conclusion from the formulation of the AmB loaded to PLGA-PEG and physicochemical characterizations of the newly formed AmB-NPs, is the selection of the best following formulations (A6, B6, C6, C7 and D6) according to the more particle size reduction occurred and maximum drug content with best drug dissolution obtained from these newly formulations. Although the ingredients are the same for all newly formed formulations, except the type of the polymer used, the best of all these formulation is the C6 (RGPd 50155) consisting from diblock PLGA-PEG copolymer with 15% PEG. The high percentage of the PEG (15 %) may contribute to this result in comparison to other used polymer have 5 and 10 % for the D and A polymer, respectively.

All tested formulations have shown slow initial release with 24.8 % in AmB loaded to PLGA-PEG polymer A3(RGPd 50105) to 61% in AmB loaded to PLGA-PEG polymer C6(RGPd 50155) release within 24 h in phosphate buffer containing 2% sodium deoxycholate. The release rates of AmB from these formulations were best fit to the Korsmeyer–Peppas model followed by Higuchi model for 24 h profile. These mathematical models are indications for mechanisms of drug release from the polymer. Therefore, a diffusion release model is the best to characterize the *in vitro* release of AmB from these polymer formulations. Korsmeyer Peppas constant 'n' = 0.245 which is the beyond the limits of Korsmeyer Peppas model. It cannot be predicted clearly. The mechanism of the drug release from the polymer may be explained as complex mechanism of swelling, diffusion and erosion.

A second method of AmB drug determination has been applied in this study for *in vivo* evaluations, a new selective, sensitive and precise liquid mass spectroscopy (LC MS/MS) method was developed to measure AmB concentrations after oral and parenteral administration of selective formulations. An excellent relationship has been obtained with the absorbance of the AmB and the concentrations used (100-4000 μg/ml) throughout the study. The precision and accuracy of the newly developed LC MS/MS method show no significant difference among inter- and-intra-day analysis ($P > 0.05$). Linearity was observed over the investigated range with correlation coefficient, $R^2 > 0.9943$ (n = 6 day). The assay was able to detect all the drug concentrations for pharmacokinetics and bioavailability estimation after oral dosing of AmB loaded to PLGA-PEG compared to its parenteral administration in rats.

Fungizone® (commercially available iv dosage form of AmB), was administered to the rats as orally dose 10 mg/kg as single dose. Detectable low AmB concentrations obtained. When AmB loaded to PLGA-PEG (C6) orally administration, at similar dose (10 mg/kg to rats, a statistically significant higher AmB concentrations was observed. Moreover administration more pronounced significant higher AmB concentrations were obtained after C6 administered as parenteral dose even it is one tenth of the dose (1 mg/kg). Both oral and parental administration showed than AmB were rapidly absorbed, distributed, and then slowly eliminated in a two compartment open model.

Glycyrrhizic acid (GA) it is a natural absorption enhancer, it is approved by many published literatures to increase the absorption of many drugs when added during the formulation procedure or added just prior to oral dose administration. The addition of GA to oral administration of C6 formulation showed significantly ($P<0.05$) increased AmB AUC and C_{max} (2 - 3 folds) in comparison to oral administration of Fungizone® at similar dose 10 mg/kg. There was no significant change ($P=0.09$) in adding GA during formulation or just mixing before oral administration. A tremendous improvement of AmB oral absorption of C6 in rats by > 790 % than Fungizone® is observed after GA Addition.

AmB is known to be nephrotoxic, it is very important to screen the *in vitro* and *in vivo* toxicity of the selected AmB novel oral formulations (A6, B6, C6, C7 and D6)Fungizone® was used as the reference standard for comparison. The toxicity tests including several *in vitro* assays*;* hemolysis, antifungal activity and analysis of the cell viability by 3-(4,5dimethylthiazoly)-2,5-diphenyl-tetrazolium bromide (MTT) assay. AmB loaded to PLGA-PEG of the all selected formulations showed a significant reduction ($P< 0.05$) in the *in vitro* hemolytic activity as compared to Fungizone®, Both C6 and C7 showed the lowest hemolysis toxic level. On another hand, AmB loaded to PLGA-PEG showed a potent activity against the *in vitro C. albicans* with a parallel decrease in the cytotoxicity against in the *in vitro* tested cell lines (Jurkat, HL60 and B-13/H cells) after exposure to 125, 250, and 500 ng/ml of AmB or its equivalents.The data obtained showed variations in cell viability after testing different formulations, most of them were likely to be safe for the used cell lines, since in all cases > 80% of the three cell lines remained viable, even at higher AmB concentration (500 ng/ml). Moreover, these data showed a dose-dependent reduction in viability (with increasing the dose there is greater loss of viability). It can be concluded that AmB-loaded to PLGA-PEG copolymer NPs of selected formulations were less toxic than the reference standard Fungizone®.

Histopathological evaluations were done after iv multiple administrations of 1.0 mg/kg body weight of the selected AmB-NPs formulation (A6, B6, C6, C7 and D6) in comparison with Fungizone®. No significant abnormalities were observed in rats given iv administration of AmB-NPs of the selected AmB novel oral formulations (A6, B6, C6, C7

and D6) except after Fungizone® administration which showed significant necrosis of the liver and the kidney.

The *in vivo* nephrotoxicity and histopathological evaluation of AmB were performed in both rats' kidneys and liver using forty eight rats divided into eight groups after single and multiple iv administrations of 1.0 mg/kg of the selected AmB-NPs formulation (A6, B6, C6, C7 and D6) in comparison with Fungizone® for one week. AmB induced nephrotoxicity was also estimated in these rats by measuring blood urea nitrogen (BUN) and plasma creatinine (PCr). A significant ($P<0.05$) nephrotoxicity was observed after Fungizone® administration compared to all AmB loaded to PLGA-PEG selected formulations (A6, B6, C6, C7 and D6). The histopathological study of the isolated kidney tissues confirmed this finding. Furthermore, the incidence of the liver toxicity due to the selected AmB loaded PLGA-PEG formulations has been reduced significantly as investigated through *in vivo* analysis (histopathological and biochemical tests) in comparison with Fungizone®.

In conclusion, a successful novel AmB oral formulation were developed using dicblock PLGA-PEG polymer with improved solubility, bioavailability, efficacy and with less toxicity to the kidney and the liver. C6 (RGPd 50155)which consist of the diblock PLGA-PEG with 15% PEG was the best tested formulation. Further investigations are needed in the near future for this formulation to be in the market as an oral delivery system of AmB.

Chapter VI. Further Work

Although the enlargement of development of new antifungal agents, AmB remains one of the most effective drug in the treatment of systemic fungal infections. The most serious adverse effects associated with AmB therapy is nephrotoxicity with sodium depletion. Several approaches have been studied to prevent these side effects. Loading AmB in liposomes or various lipid formulations seems to be the best strategy to reduce its nephrotoxicity and improve drug delivery to target sites. While these formulations are proven to be effective, they still used as parenteral administrations and need of certain safety issues associated with this administration and high drug cost.

An oral formulation of AmB is appropriate to treat infections and provide or prophylactic therapy for high-risk patients. This PO route will also increase compliance and prevent parenteral side effects.

Several strategies can be considered for enhancing the anti-leishmanial activity of oral AmB-NPs. Enhancing particle uptake by macrophages. The particle uptake by macrophages is mostly dependent on two main factors, the particle size and the hydrophobicity/hydrophilicity. Increasing the particle size can improve the particle uptake by macrophages; however, it adversely affects its intestinal uptake. Altering the hydrophobicity of the particles may enhance the particle opsonization and subsequent uptake by macrophages. The other strategy could be the altering of release profile of AmB-NPs. Increase in rate of AmB release from AmB-NPs can increase the concentration of free AmB available for killing the parasites, which may lead to improved anti-leishmanial activity. PLGA-PEG is a copolymer, made up of polylactic glycolic acid and polyethylene glycol. As PLGA being more hydrophobic than PEG, hydrophobicity and degradation rate of PLGA mainly depends on the molar ratio of lactic and glycolic acid in the polymer chain. By varying copolymer composition of PLGA, degradation rate (and thus drug release) or hydrophobicity of the AmB-NPs can be optimized to obtain higher antileishmanial activity. On the other hand, NPs can actively be targeted to macrophages by functionalizing the particles using specific ligands.

Preliminary studies have shown that freeze-dried AmB-NPs loaded by PLGA-PEG showed significant reduction in the MPS, enhanced the DEE and high yield obtained. The SEM and TEM images indicate that the presence of both isolated and agglomerated

particles of spherical shape (in the nanometer size range). The results of the FTIR show sort of interaction between the drug and the polymer, which suggest the loading of the into the polymer. In addition to SEM, TEM and DSC further physicochemical confirmation can be done using X ray diffraction, show the pattern of the AmB-NPs either crystalline or amorphous state. Moreover, atomic force microscopy (AFM) displays the three dimension image of the AmB-NPs and can confirm the size distribution of the particle size for the new formulations.

Furthermore, additional studies can be carried on different addition of excipient as and different concentration to investigate its effect on the AmB-NP formulations. Additionally stability test (at room temperature and accelerated stability test) should be done the for the new formulation on short and long term stability studies.

A simple, fast, accurate and reliable analytical HPLC method for measuring AmB in *in vitro* was developed during this study. It is sensitive for AmB, can measure as low as 250 ng/ml, under the assay conditions, the retention times for AmB and IS were around 2.8 and 4.2 min, respectively.

The release profiles indicate biphasic release of AmB from PLGA-PEG-NPs formulations. In the first phase an initial rapid release of about 21-51%, depending on the formulation, followed by a slower phase, after 6 h, where only about 11% was released. Further studies are suggested to assess the stability of oral AmB loaded PLGA-PEG copolymer over short and long period of time.

A new developed simple, rapid and sensitive analytical UPLC Ms/Ms method was utilized and validated for the analysis of AmB concentrations in rat's plasma after administration of the AmB loaded to PLGA-PEG tested formulations as either iv or PO administration. The lower detectable concentration was 100 ng/ml at a signal-to-noise ratio of >10, with the corresponding R.S.D. of 14 %.

AmB should not be kept in the auto sampler more than overnight analysis to ensure reproducibility of the assay; it is unstable at room temperature. Exposing AmB to drastic conditions revealed that AmB is stable in acidic medium even after boiling, losing only 5.7% of its nominal value. In the meantime, both water and basic condition showed

complete loss of AmB even without boiling for all tested samples. A further study should be done , the long-term stability studies (as per ICH guidelines) should be considered to determine the influence of a variety of environmental factors such as temperature, humidity and light to establish a shelf life for the drug product and recommended storage conditions. Since the formulation is intended to be administered orally, the formulation should be essentially palatable. To improve the palatability, various additional excipients such as sweetening and flavoring agents need to be incorporated in the formulation. Since these additives can affect the stability of the formulation, their effects on the stability of the final formulation should be evaluated.

The present studies demonstrated pharmacokinetic proof of concept in feasibility of the oral delivery of AmB as nanoparticulate formulation resulted in considerable intestinal absorption into the systemic circulation and with considerable therapeutic efficacy in rats. The plasma pharmacokinetics of orally administered AmB resembles those of Fungizone®-PO. The addition of GA significantly improve the bioavailability of AmB, unexpectedly, the GA addition just prior to oral administration or combined it during the formulation process does not significantly affect the bioavailability. Further study should be done on administration of AmB tested formulations for more than 24 h, multiple doses and of large number of rats, since the pharmacokinetic of the AmB still unclear.

The present study also investigates the selected AmB-NPs loaded to PLGA-PEG for *in vitro* and *in vivo* toxicity tests. The *in vitro* and *in vivo* studies done for the selected AmB loaded to PLGA-PEG nanoparticles resulted in significant lower side effects especially nephrotoxicity in comparison to Fungizone®. No adverse effects were identified in liver and kidney *in vivo*, as it is shown by histopathology analysis and quantification of serum biochemical parameters. Additionally nanoparticles did not induce hemolysis. Furthermore, the prepared AmB-NP, have high therapeutic efficacy and are can be useful for the treatment of fungal infection including candidiasis. Together these results demonstrate the novel oral tested AmB-NPs loaded with PLGA-PEG formulation could be a promising oral dosage form of AmB for the treatment of fungal infections.

Further studies is suggested to assess the antifungal activity of oral AmB loaded PLGA-PEG copolymer over short and long period of treatment to assess both antifungal activity and survival of the animals upon the long treatment.

The overall physicochemical tests for its pharmacokinetics and *in vitro* and *in vivo* cytotoxicity studies showed that PLGA-PEG copolymers were a safe excipient as well as an alternative carrier for oral drug delivery for AmB.

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