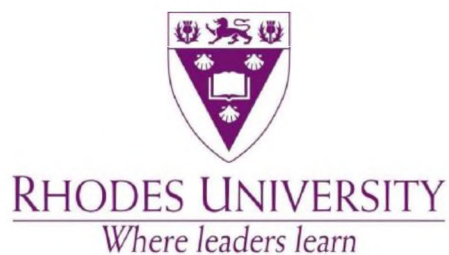


Inhalable Particulate Systems for Anti-Tubercular Drug Delivery

A thesis submitted in fulfilment of the requirements for the degree of

Master of Science (Chemistry)

At



By

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DEDICATION

This thesis is dedicated to my late father Mr Stewart Nkanga Isalomboto, the one who would be the happiest ever to see me growing academically; may the earth lay over him gently.

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ABSTRACT

Tuberculosis (TB) is a deadly infectious microbial disease that is currently dominating public health concerns. Among the pharmacological issues in the management of TB are the poor bioavailability of some anti-TB drugs, mostly due to the fast first-pass metabolism, and high drug load needed for combination therapy. These result in a lengthy treatment with several adverse effects causing decreased patient compliance. These factors often lead to the therapeutic failure and promote the development of drug resistant strains, justifying the urgent need for new therapeutic strategies. Liposomes are lipid-based particulate vehicles known to be the most clinically appointed drug carriers currently. Liposomal systems are reported to be rapidly engulfed by macrophages – where the mycobacterium often resides. This makes liposomes appropriate vehicles for targeted anti-TB drug delivery. Many research groups have reported the potential of liposomes systems to deliver anti-TB drugs. However, the costly formulation status of liposomes, due the use of expensive synthetic or highly purified natural phospholipids, is a limitation to the treatment of a poverty related infectious disease like TB. The aim of this study was to design and develop liposomes for pulmonary delivery of anti-TB drugs using crude soybean lecithin (CL) and its purified version. CL is an FDA-approved naturally occurring phospholipid mixture that is quite cheap and readily available. Various liposome batches were prepared using a film hydration method and characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM). Liposomes composed of CL and cholesterol (Chol) in a 3:1 mass ratio were selected for drug encapsulation based on the following characteristics: polydispersity index (PDI, 0.28), mean particles sizes (PS, 502 nm) and zeta potential (ZP, -56 mV). Isoniazid (INH) was encapsulated as a model drug using a freeze-thaw loading technique and an HPLC method was validated for quantitative analysis. The physicochemical properties of INH-loaded liposomes were comprehensively investigated using thermal, microscopy and spectroscopic techniques. This formulation showed a high encapsulation efficiency (%EE) of 78%, much better than the liposomes made from purified lecithin, 20%. Other characteristics of INH-loaded liposomes, which make them attractive for pulmonary TB therapy, are presented in this dissertation. These include a controlled release of 50% of the encapsulated INH over 12 hours. Finally, rifampicin (RIF) was added as a hydrophobic model drug and several evaluations were conducted on these dual drug-loaded liposomes. Of particular interest, it was noted that the dual drug-loaded liposomes made of CL alone showed the highest %EE (59% for INH and 90% for RIF) compared to those containing Chol or those made of purified lecithin. Surprisingly, the average PS of the dual CL-based liposomes (1114 nm) was in the size range reported for optimum deep lung deposition and macrophage uptake. In addition, the mean ZP of these liposomes (-63 mV) seems to be favourable for their shelf stability and internalization by macrophages. Overall, these findings show that the dual CL-based liposomes developed would be promising for macrophage-targeting pulmonary delivery of anti-TB drugs.

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CHAPTER ONE
GENERAL INTRODUCTION

1. TUBERCULOSIS

1.1. Definition and History

Tuberculosis (TB) is a deadly infectious disease caused by *Mycobacterium tuberculosis* complex (MBTC), which is part of the current public health concerns (WHO, 2016). The MBTC is a mammalian tubercle bacterium consisted of various species that cause animal and human TB infection (Cousins et al., 2003; Van Soolingen et al., 1997). The strains that affect animal and human have a common ancestor that had probably started to infect humans since the Neolithic Revolution period (Comas and Gagneux, 2009).

The prehistoric status of human TB was established based on archeological research, which detected TB traces dating from 3000 to 2500 years before the Christ in Egyptian mummies (Zink et al., 2003). According to the traditional belief, TB infection was a phenomenon related to the vampirism from dead family members. This folk opinion was abandoned when bio-archeological and bio-cultural evidences related to TB were established (Sledzik et al., 1994). In 1689, Dr Richard Morton associated the pulmonary form to the presence of tubercular bacilli in the lungs (Trail, 1970). In 1839, Schönlein JL called the disease “tuberculosis” (Zur, 1839). Since that time, the isolation of the mycobacterium was established as an efficient method for the diagnosis of a TB infection (Rothschild et al., 2001).

In 1882, Robert Koch identified and described TB bacterium; and got a Nobel Prize in Medicine for this scientific achievement. He also attempted to develop a TB treatment based on glycerin extract (tuberculin) of the tubercular bacilli. However, tuberculin was established later on as an effective mean for pre-symptomatic diagnosis of TB, as no therapeutic efficacy was observed (Waddington, 2004). In 1906, Albert Calmette and Camille Guérin used attenuated bovine MBTC strains to develop immunization against TB in humans, calling the vaccine BCG, for bacille Calmette-Guérin (Bonah, 2005). Despite the widespread acceptance of BCG, the prevalence and incidence of TB over the years indicate the failure of this vaccine at preventing the disease and show that chemotherapy is the only feasible option at the moment.

Before the introduction of antibiotics, the treatment of TB was based on the fresh air and labor in Sanatorium – that could reduce the mortality rate up to 50% – and the pneumothorax technique, which involved a surgical shrinkage of the affected lung that could heal TB lesions efficiently (McCarthy, 2001; Shields, 2009). Though the discovery of Streptomycin in 1946 decreased the mortality rate of TB, the surgical operation involving the ablation of the

affected chest cavities was established again in order to face the issues of drug resistance and multi-drug resistance encountered after the development of other antibiotics (Lalloo, 2006). In 1993, the World Health Organization has declared TB disease as a major public health problem that requires an urgent and considerable attention to be sorted (WHO, 2012).

1.2. Epidemiology

Tuberculosis is one of the top two most devastating infectious diseases in the world, second only to the human immunodeficiency virus (HIV) infection (WHO, 2016; Venturini et al., 2014). Every year, around 1 % of the global population contracts *M. tuberculosis* (WHO, 2002), among which only 5 to 10 % develop active TB disease (Skolnik, 2011). Up to 8.8 million of new TB cases were recorded in 2010, along with 1.20 to 1.45 million deaths occurring mainly in developing countries (WHO, 2011; Lozano, 2012). The World Health Organization reported about 10.4 million of new active TB cases with 1.8 million deaths in 2015, among which over 95% occurred in developing countries (WHO, 2016).

While the incidence of TB infection has significantly reduced between 2004 and 2014, and new TB cases decreased by 17 % in this time interval (Kielstra, 2014), TB is still a common disease in many countries. In Africa and Asia, approximately one TB infected person dies every twenty seconds (Piccini et al., 2014; Dartois, 2014). In addition, up to 80 % of Asian and African populations give positive tuberculin skin test, compared to only 5 to 10 % recorded in the United States of America (Kumar et al., 2007). Considering the worldwide distribution of TB cases rated per 100,000 populations in 2010, TB infection could affect 332 individuals in Africa, 278 in Southeast Asia, 173 in Eastern Mediterranean, 139 in Western Pacific, 63 in Europe, 36 in America and 178 globally (WHO, 2011). **Figure 1.1** presents the global geographical distribution of new TB cases in 2015. Overall, the map shows that Sub-Saharan Africa and Asia represent the most TB affected areas along with South America and Eastern Europe that run behind (WHO, 2016).

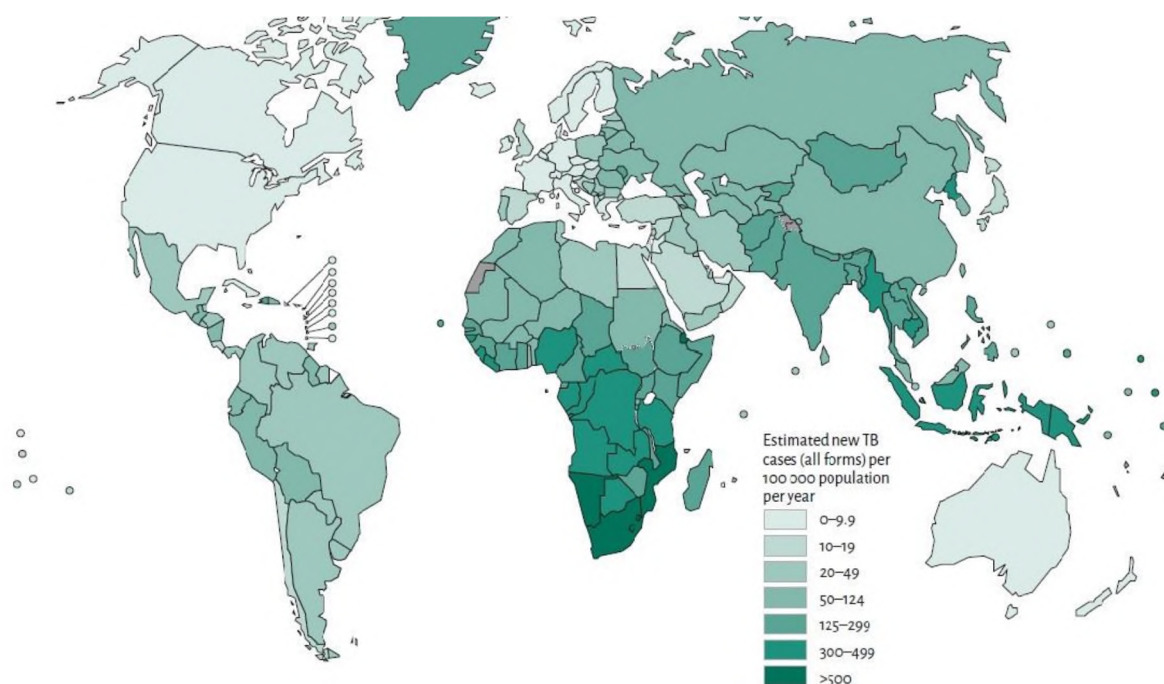


Figure 1.1: Worldwide geographical distribution of new TB cases rated per 100,000 populations in 2015 (WHO, 2016).

1.3. Pathogen

The causative microbial agent of TB, *Mycobacterium tuberculosis* complex (MBTC), is an aerobic bacillus consisted of very similar *Mycobacterium* species. These include *M. tuberculosis* that causes human TB and rarely *M. canetti*, as well as *M. bovis*, *M. africanum*, *M. microti* and *M. pinnipedii* that cause TB in domestic and wild animals; and *M. bovis* Bacillus Calmette-Guérin (BCG), which is currently used as TB vaccine strain (Cousins et al., 2003; Moda et al., 1996; Soolingen et al., 1997). Additionally, the *Mycobacterium* genus embraces also *M. avium* complex (MAC) that comprises two closely related non-tubercular opportunistic bacteria, *M. avium* and *intracellulare*. These mycobacteria affect immunocompromised patients commonly in developed countries; while *M. tuberculosis* worldwide affects both people living with the human immunodeficiency virus (HIV), and non-HIV infected ones (Sevilla et al., 2015; Thegerström et al., 2012; Sansila et al., 1998). To some extent, this suggests a possible MBTC–MAC coinfection and justifies the urgent need to deal with potential therapeutic strategies, such as the use of effective drug delivery systems.

MBTC is visualized by microscopic observation in sputum of a TB infected individual. Its characterization is based on its high lipid contents and mycolic acid in the cell wall, which causes it to weakly stain Gram-positive or to not retain the dye at all. Though host cells are

required for their natural replication, *in vitro* cell culture of MBTC is possible. However, the rate of division of MBTC is very slow, 16 to 20 hours, as compared to that of other bacteria that typically take less than 1 hour (Madison, 2001; Parish and Stoker, 1999).

1.4. Pathogenesis and Symptoms

Tuberculosis is initiated by the entry of tubercle bacilli in the form of aerosol droplets that other patients have expelled while coughing or sneezing. The presence of *M. tuberculosis* in respiratory alveoli mostly leads to asymptomatic TB infection, also known as latent TB infection (LTBI) that occurs in an estimated 90% of cases. Only 10% of TB infected people suffer from active TB disease (ATBD) with several clinical manifestations. Without an urgent anti-TB therapeutic regimen, there is only a 44% of chance of survival for a patient with ATBD. In case of low immunity, such as HIV infected patients, the risk that a LTBI may evolve to ATBD increases by 10% per year (Kumar et al., 2007; Mainous and Pomeroy, 2009).

The starting point of a TB infection is the replication of the bacteria in the endosomes of alveolar macrophages. These immune cells identify the mycobacterium as a foreign particle and try to remove it from the body via the process of phagocytosis. The cell produces antimicrobial proteases, lipases, reactive oxygen and nitrogen species and a highly acidic environment in an attempt to kill the infectious agent in the phagolysosomes. The process of phagosome maturation, which is the fusion of phagosomes (containing the mycobacterium) and lysosomes (containing toxic chemicals mentioned above) into phagolysosomes, aims to degrade and destroy the pathogens. Despite these series of actions, *M. tuberculosis* resides and replicates inside of the infected immune cells due to its protective cell wall that contains mycolic acid. In addition, *M. tuberculosis* interferes with the maturation of phagosomes into phagolysosomes and increases the pH of endosomes (Hawn et al., 2013; Kumar et al., 2007; Houben et al., 2006).

The infected macrophages are surrounded by lymphocytes, fibroblasts and other macrophages, giving a huge biological aggregate called a granuloma. Though the formation of granulomas tends to prevent the mycobacteria from spreading in the body, attack by the immune system seems to be compromised as the macrophages and dendritic cells cannot present the antigens to lymphocytes (Crowley, 2010). Typical symptoms of tuberculosis include chills, fever, and loss of appetite, night sweats, weight loss, nail clubbing and fatigue

(Gibson et al., 2005). **Figure 1.2** presents the principle clinical manifestations of the variants and stages of tuberculosis.

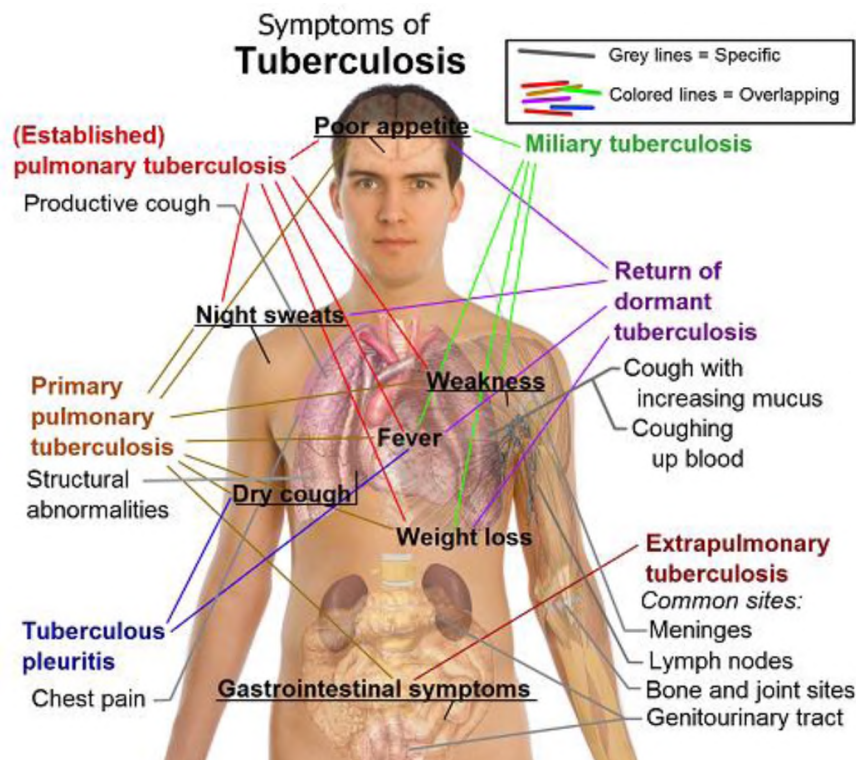


Figure 1.2: Major symptoms of tuberculosis with multiple variants at different stages.
http://tlangsam.blogspot.co.za/2014/06/kum-3-sung-manipur-ah-tb-leia-thi-mi-224_30.html
 (Accessed on 19/10/2016).

In 90% of active TB cases, the infection involves the lungs to give what is known as pulmonary TB; while in the remaining TB cases the infectious agent affects the other organs of the body giving extra-pulmonary TB. The chest pain and chronic sputum-producing cough represent the main signs of pulmonary tuberculosis (Behera, 2010; Lawn and Zumla, 2011). In case of damage to the tissues, the bacteria enter the blood stream and cause miliary TB that represents around 10% of extra pulmonary TB cases. This disseminated TB develops commonly in children and HIV infected patients, and represents a severe deadly form of active TB infection with 30% of the fatalities (Harries, 2005; Jacob, 2009). Pleura, the central nervous system, lymphatic system, genitourinary tract, skin, bones and joints are also the potential sites of extra pulmonary TB, respectively named tubercular pleurisy, tubercular meningitis, scrofula of the neck, genitourinary TB, tubercular ulcer and osseous TB or tubercular osteomyelitis (Kabra and Vimlesh, 2006; Golden and Vikram, 2005).

1.5. Diagnosis

The diagnosis of active TB disease is often based on pulmonary manifestations lasting for 14 days. The outcomes of chest radiological evaluations and acid-fast bacilli via cell cultures serve as the foundation of the diagnosis. The resulting microscopic detection of *M. tuberculosis* in the biological sample, such as sputum, pus or tissue biopsy; is the most frequently used diagnosing evidence despite the fact that the sensitivity of this test does not exceed 60% (Bento et al., 2011; Piccini et al., 2014). The identification of mycobacteria is often achieved by means of Ziehl–Neelsen and Kinyoun stains, which represent the most commonly used staining techniques. Other techniques include auramine–rhodamine staining and fluorescence microscopy. As the rate of division of *M. tuberculosis* is very slow, the treatment of TB is sometimes initiated before the results of cell cultures based on the clinical signs observed (Madison, 2001; Parish and Stoker, 1999).

Nucleic acid amplification tests and adenosine deaminase are the fastest diagnostic tests for TB, but not recommended that much as they can interfere with ongoing treatments (Bento et al., 2011). The tuberculin skin test and interferon gamma release assays are often used to diagnose latent TB infection and rarely active TB disease in highly exposed populations, mostly in developing countries. The combined results from the two tests are more reliable than each test considered separately. However, the two tests may generate false positive readings in case of HIV or patients infected by other *Mycobacterium* species (Chen, 2011; Jindal, 2011; Sester, 2011).

1.6. Therapeutic management

The management of TB relies on vaccination, early detection of the mycobacterium in highly exposed individuals, such as households and workmates, and chemotherapy (Hawn et al 2014; Harris, 2013). To date, BCG is the most common therapeutic regimen used for prevention of TB. This vaccine has been reported to decrease the risk for paediatric MBTC infection by 20% and the development of latent TB infection (LTBI) to active TB disease (ATBD) by 60% (Roy et al., 2014). Though BCG is a widespread vaccine, its use is associated with some concerns such as its time-dependent protective effect, as the immunity can be lost after 10 years (Lawn and Zumla, 2011). Additionally, this vaccine is reported to generate false positive tuberculin skin test (Teo and Shingadia, 2006), which is commonly used for preventative screening (Hawn et al., 2014; Harris, 2013).

The curative treatment of TB is based on the use of antimicrobial agents to kill the infectious agent. Access of some drugs to mycobacteria is compromised by the protective function of the cell wall that reduces the effectiveness of the treatment (Brennan and Nikaido, 1995). Generally, the anti-tubercular regimens used to treat LTBI and ATBD in adults are similar to those used in children. LTBI is treated by isoniazid (INH) and rifampicin (RIF) taken as either a mono therapy, which should be discouraged due to possible resistance development, or a bi-therapy to prevent further development of active TB disease. The administration of INH to LTBI individuals for 9 months offers 90% to 100% of cure rate (Piccini et al., 2014). The treatment can unfortunately be associated with serious side effects of INH, such as INH-induced hepatitis that can evolve to a deadly acute hepatic failure. Some benign adverse effects such as nausea, headache, rashes, hepatitis and peripheral neuropathy can also appear. The duration for RIF-based regimen is 4 months for adults and 6 months for children. The treatment based on INH and RIF combination takes 3 months and provides 60% of the cure rate for LTBI in adults, but the effectiveness of this regimen in children is not yet proven (Piccini et al., 2014).

The two major antimicrobial agents for ATBD include INH and RIF, which are used in combination with pyrazinamide (PYZ) and ethambutol (ETM). These four drugs compose the current first-line anti-tubercular regimen, where streptomycin (STR) replaces ETM, which is sometimes contra-indicated in children due to its possible ocular toxicity. Another first line anti-TB agent is rifabutin (RFT) that replaces RIF in some cases. The treatment of ATBD starts with intensive daily administration of all the first-line anti-TB drugs for two months. Then, the combination of INH and RIF is kept for 4 months to complete the 6 months anti-TB regimen (Piccini et al., 2014; Nuermberger et al., 2010). This lengthy drug administration causes various adverse effects that decrease significantly the compliance of the patient to the established anti-TB regimen, leading to both the therapeutic failure and the development of drug resistant strains (Hawn et al., 2013; Yuen et al., 2013).

The emergency of mycobacteria strains resistant to the first-line anti-TB drugs led to the establishment of a second-line of antimicrobial agents for multi-drug resistant TB. These include ethionamide or prothionamide, kanamycin, amikacin, terizidone, cycloserine, capreomycin, viomycin, para-aminosalicylic acid and fluoroquinolones such as ofloxacin, levofloxacin and moxifloxacin (Kumar et al., 2011). The treatment with these drugs is however associated with several side effects that limit their use in pediatric populations, where there are not yet enough therapeutically related safety data. Additionally, the lack of

pediatric dosage forms for the second-line anti-TB drugs exposes children to possible inappropriate dosing; sub-therapeutic or toxic drug administration (Piccini et al., 2014). This demonstrates the need for novel drugs and drug delivery systems in order to improve the therapeutic management of TB. **Table 1.1** presents the first-line and second-line anti-TB drugs along with their unit doses and aqueous solubilities (adapted from Sosnik et al., 2010).

Table 1.1: First- and second-line anti-TB drugs with their unit doses and water solubility values.

Designation	Compound name	Unit dose (mg)	Water (mg/ml)	Solubility
First-line TB drugs	Isoniazid	300	125	
	Rifampicin	300	1 – 2	
	Pyrazinamide	500	14	
	Ethambutol	400	100	
	Streptomycin	500	> 20	
	Rifabutin	150	0.19	
Second-line TB drugs	Clofazimine	100	0.01	
	Ethionamide	500	0.1	
	Clarithromycin	500	0.00033	
	p-Aminosalicylic acid	500	1.7	
	Cycloserine	500	100	
	Amikacin	1000	Unavailable	
	Kanamycin A	1000	Unavailable	
	Capreomycin	1000	Soluble	
	Levofloxacin	500	Sparingly soluble	
	Moxifloxacin	400	Unavailable	
	Gatifloxacin	400	60 mg at pH 4	
	Linezolid	400	Soluble up to 3mg	

1.7. Anti-TB drugs profiles

In this work, the two major first-line anti-TB agents were chosen as model drugs for encapsulation study, namely INH and RIF. The reasons behind the selection of these two

drugs were: i) their importance in the current therapeutic regimen, as they are clinically established for the treatment of all the forms of TB; and ii) their respective very high and low solubility in aqueous media as shown in **Table 1.1**.

1.7.1. Isoniazid

1.7.1.1. Physicochemical aspects

Molecular formula: C₆H₇N₃O

Molecular weight: 137.14 g/mole

Structure: The molecular structure of isoniazid corresponds to a hydrazide of isonicotinic acid presented in **Figure 1.3**.

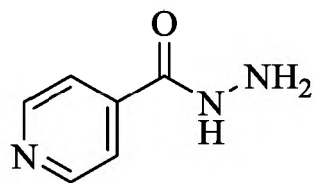
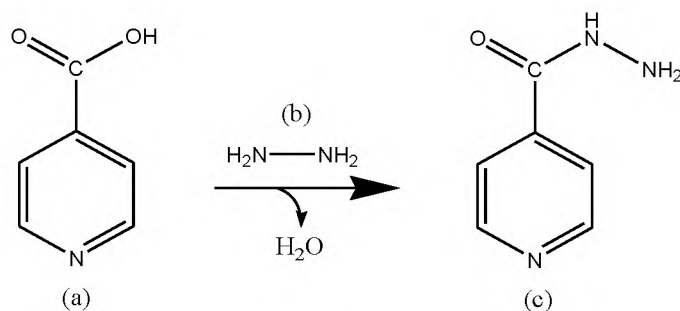


Figure 1.3: Molecular structure of Isoniazid.

Systematic name (IUPAC): 4-Pyridinecarboxylic acid hydrazide; Pyridine-4-carbohydrazide.

Synonyms: Isonicotinic acid hydrazide, isonicotinyl hydrazine, INHA, INH.

Synthesis: Two synthesis pathways are proposed for the production of INH. The first method requires the conversion of isonicotinic acid (a, **Scheme 1.1**) into its ethyl or methyl esters that react with hydrazine (b, **Scheme 1.1**) at 70°C to produce INH. The second synthetic strategy consists in reacting directly isonicotinic acid and hydrazine in a one-step method, which is recommended due to lower temperature and ease of purification (Vrabete and Modra, 2013).



Scheme 1.1: Pathway for INH synthesis (Vrabete and Modra, 2013).

Organoleptic characters: INH is a white to yellowish crystalline powder that is very soluble in water and scantily soluble in alcohol (BP, 2013).

Melting point: 170°C to 174°C (BP, 2013).

1.7.1.2. Pharmacological aspects

Drug category: Anti-tubercular agent

Available dosage forms: Oral solutions, tablets, intra-muscle and intravenous injections.

Daily dose: 4 to 5 mg/kg of weight for adults and 5 to 10 mg/kg of weight for children.

Mechanism of action: INH is a prodrug that is activated by a mycobacterial enzyme called catalase peroxidase (KatG). Inside of the *Mycobacterium tuberculosis*, KatG couples the isonicotonic acyl of INH to NADH. The biological activity of INH is based on the formation of this isonicotinic acyl-NADH complex that blocks the fatty acid synthase of the mycobacterium. This leads to the inhibition of mycolic acid synthesis that causes disruption of cell wall and cell death. Additionally, INH produces active free radicals by inhibiting the cytochrome P450 system. INH shows bactericidal or bacteriostatic activities, depending on whether the bacterial strain involved grows fast or slowly. INH is mainly active against *M. tuberculosis*. Its effectiveness against *M. kansasii* and *M. xenopi*, which are atypical species, has been also reported (Ahmad et al., 2009; Suarez et al., 2009; Benfang et al., 2000; Berning and Peloquin, 1998).

Metabolism: The metabolism of INH in the liver involves an enzymatic acetylation. Its half-life is 0.5–1.6 hours for fast acetylators and 2–5 hours for slow acetylators. The metabolites of INH are eliminated via urinary system, but no dose adjustment is required in case of kidney failure. The maximum protein binding for INH is about 10 %. Being an inhibitor of cytochrome 2C19 and 3A4, INH slows down the metabolism of some drugs, such as carbamazepine, theophylline, valproate and ketoconazole, and exposes the patient to any possible toxic effects due to the accumulation of these drugs. INH is an enzyme inducer for acetaminophen, which is converted into toxic forms when administered along with INH (Bass et al., 1994; Baciewicz, 1993; Fleenor et al., 1991; Jonville et al., 1991; Burk et al., 1990; Murphy et al., 1990).

Indications: INH is used in combination with other first-line anti-TB drugs to treat latent and active pulmonary and extra-pulmonary TB infections (Piccini et al., 2014; Nuermberger et al., 2010).

Contraindications: severe hypersensitivity to INH, drug-induced hepatitis and liver failure.

Side effects: High liver activity and drug toxicity, peripheral neuropathy, nausea and vomiting, aplastic anemia, thrombocytopenia, agranulocytosis, neuropathy and deficiency of vitamin B6 (Steichen et al., 2006; Yee et al., 2003; Bothamley, 2001; Schaberg et al., 1996).

Marked names: Cotinazin®, Ditubin®, Hydra®, Hyzyd®, Isovit®, Laniazid®, Nydrazid®, Stanozide®, Rimifon®, Rifater®, Rifinah®, Afracin®, 3-FD® and 4D®.

Other use: Chromatographic discrimination of ketones via formation of isonicotinyl hydrazone derivatives that are then characterized by their bathochromic shift (Fensk, 2008 & 1998).

1.7.2. Rifampicin

1.7.2.1. Physicochemical aspects

Molecular formula: $C_{43}H_{58}N_4O_{12}$

Molecular weight: 822.95 g/mole

Structure: Rifampicin is a heterocyclic macromolecule that contains a naphthoquinone nucleus, which is extended by means of an aliphatic chain as shown in **Figure 1.4**. The naphthol core and hydroxyl groups of rifampicin are the main functional groups involved in its biological activity, inhibition of RNA polymerase of the pathogens (Campbell et al., 2001).

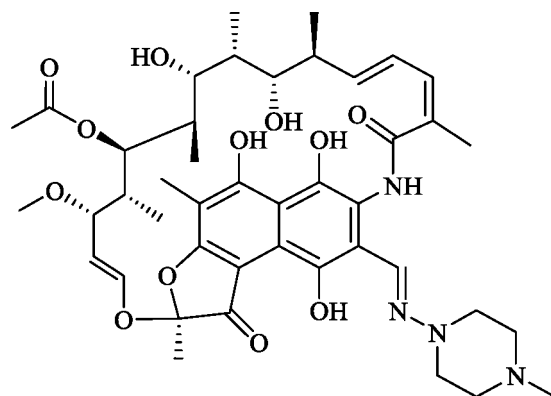


Figure 1.4 : Molecular structure of Rifampicin.

Systematic name (IUPAC): (2S, 12Z, 14E, 16S, 17S, 18R, 19R, 20R, 21S, 22R, 23S, 24E)-5, 6, 9, 17, 19-Pentahydroxy-23-methoxy-2, 4, 12, 16, 18, 20, 22-heptamethyl-8-[[[4-thylpiperazin-1-yl)imino]methyl]-1,11-dioxo-1,2-dihydro-2,7-(epoxypentadeca [1, 11, 13] trienimino) naphto[2,1-b] furan-21-yl acetate.

Synonyms: rifaldazine, rofact, rifampin, rifamycin AMP, RMP, RA, RF, and RIF.

Production: RIF is produced by grafting 3-(4-methyl-1-piperazinyl)-iminomethyl on the naphthol core of rifamycins SV, which are naturally occurring antibiotics produced by the bacterium *Ammycolatopsis rifamycinica*. Rifamycins SV are considered as ansamycins, due to the presence of aliphatic ansa chain that stretches the naphthol nucleus. Other antibiotics belonging to rifamycins family are rifabutin, rifapentine and rifalazil (Bennett, 2015; McHugh, 2011).

Organoleptic characters: RIF is a light sensitive crystalline powder that appears reddish to brownish due to the large conjugation system of its naphthoquinonic nucleus.

Melting point: 183°C to 188 °C.

Stability in acidic medium: RIF gets degraded into 3-formyl rifamycin in acid medium. Though reversible, this decomposition is promoted by INH that reacts with 3-formyl rifamycin to produce a isonicotinyl hydrazone derivative (Singh et al., 2000; Shishoo et al., 1999).

1.7.2.2. Pharmacological aspects

Drug category: Anti-tubercular agent

Available dosage forms: Oral solutions, capsules, tablets and intravenous injections.

Daily dose: 8 to 12 mg/kg of weight for adults and 10 to 20 mg/kg of weight for children.

Mechanism of action: RIF inhibits the synthesis of bacterial proteins by blocking the RNA polymerase of the pathogens. This steric interaction occurs specifically at the subunit beta of this DNA-dependent bacterial enzyme located in between DNA and RNA strands. Like this, RIF prevents the formation of phosphodiester bonds from occurring within the nascent RNA. RIF is effective against the mycobacteria – especially *mycobacterium tuberculosis*, *M. avium complex* and *M. kansasii* – and some other non-mycobacterium species such as methicillin-resistant *Staphylococcus aureus* and *Chlamydia pneumoniae* (Feklistov et al., 2008; Campbell et al., 2001; Gieffers et al., 1998).

Metabolism: RIF is mainly metabolized in bile via hydrolysis by esterases that remove its acetyl group. Though still bactericidal, the deacetylated RIF is eliminated in feces due to its lack of ability to be re-absorbed. The half-life of RIF, 2–5 hours, is dependent on the administered doses and food consumption that can reduce significantly its intestinal absorption. RIF is the most potent inducer of the enzymes belonging to the family of cytochrome P450 (CYP450). In case of co-administration with RIF, all the drugs that are metabolized by CYP450 show very low plasmatic concentrations and their efficacy is compromised. The enzyme inducer effect of RIF affects the activity of warfarin, everolimus, atorvastatin, caspofungin, clarithromycin, rosiglitazone, celecoxib, voriconazole, lorazepam, pioglitazone, gentamicin and amikacin as well as antiretroviral drugs (Riss et al., 2008; Stockley, 1994). This indicates the need for better drug delivery systems in order to limit drug–drug interactions commonly encountered, especially in the case of HIV–TB coinfection.

Indications: RIF is used in combination with other first-line anti-TB drugs to treat latent and active pulmonary and extra-pulmonary TB infections. RIF is also used in the treatment of some non-tubercular diseases such as Hansen's disease (leprosy), where it is associated with clofazimine and dapsone, and other bacterial infections caused by RIF-sensitive pathogens (Piccini et al., 2014; Sharma et al., 2013; Sosnik et al., 2010; Aboltins et al., 2007).

Contraindications: hypersensibility to RIF, HIV therapy with anti-proteases and any other therapy based on drugs metabolized by CYP450 enzymes.

Side effects: liver toxicity and failure, fever, headache, rashes, hyperpigmentation, gastrointestinal disturbances causing nausea, vomiting, abdominal cramps, diarrhea, arthralgia, immunological reactions, reddish to brownish biological fluids, some

breathlessness, chills and malaise (Pugazhenthian et al., 2015; Yee et al., 2003; Schaberg et al., 1996).

Marked names: Rifater®, Rimifon®, Rifadin®, Abrifam®, Rifaprodin®, 3-FD®, 4D®.

Other use: RIF is used in Biotechnology to block the RNA polymerase of the bacterial host cell in order to promote the expression of recombinant proteins (Jan et al., 1999; Arcidiacono et al., 1998).

1.8. HIV-Associated Tuberculosis

Tuberculosis and human immunodeficiency virus (HIV) infection represent the top two most devastating infectious diseases in the world. Recently, the world health organization estimated around 2 billion of latent TB infected individuals and 35.3 million of HIV infected patients worldwide. Of 10.4 million of latently TB infected people who developed active TB in 2015, 0.4 million were patients living with HIV. This statistic sounds serious considering 2.5 million new HIV cases reported annually (WHO, 2016). Due to the heavy burden recently reported, around 78%, Sub-Saharan Africa has been considered to bear the brunt of the dual epidemic (WHO, 2016; Venturini et al., 2014). The incidence and prevalence of these two threatening diseases appear to be linked whilst their pathological synergies are deemed multi-factorial and not still completely understood. While HIV strongly increases the susceptibility of new or latently TB infected people to develop the active form of the disease, TB causes the death of around 35% of the individuals living with HIV infection (WHO, 2016; Venturini et al., 2014). Previously, the decrease in the CD4 T-cells was correlated with a high susceptibility in developing active TB disease for HIV-positive latently TB infected individuals. However, some recent investigations have demonstrated that even people with high CD4 T-cell counts, such as those under effective antiretroviral treatment or after HIV seroconversion, could still show high risk of developing active TB disease in endemic TB areas (Geldmacher et al., 2012; Sonnenberg et al., 2005).

According to the guidelines established by WHO, the diagnosis of TB associated with HIV should be conducted as the case with HIV negative patients. The common clinical characteristics such as longstanding cough, weight loss, night sweat, fever, etc.; and the results of biological tests, for instance tuberculin skin and radiological tests, should be considered for diagnostic purposes (Venturini et al., 2014). The treatment of HIV-TB coinfection is initiated by an early administration of the four first-line anti-TB drugs, INH, RIF, PYZ and ETM; for 2 to 8 weeks before starting with anti-retroviral drugs (ARV). This is

due to the possible drug–drug interactions between the antimicrobial agents involved. As an important CYP3A4 inducer, RIF reduces significantly the plasmatic concentrations of nevirapine and some HIV protease inhibitors, which are known to be metabolized by enzymatic proteins (like CYP450), and cause antiviral therapeutic failure. To avoid such a consequence, the two therapies must be initiated consecutively in order to render TB tolerant to the patient so that the dose adjustment or RIF removal may be possible before administering ARV. Another drug belonging to rifamycins group as RIF, rifabutin (RFB), is used in combination with ARV. Although RFB is a less potent CYP3A4 inducer, its limited availability and restricted antimycobacterial spectrum, as compared to RIF, is a concern for the current clinical conditions (Venturini et al., 2014). Therefore, novel therapeutic strategies, such as the use of effective drug delivery systems, are required in order to control such pharmacological issues, considering that HIV patients are mostly susceptible to MBTC and other opportunistic pathogens such as MAC.

2. PULMONARY DRUG DELIVERY

2.1. Respiratory system

2.1.1. Anatomical description

The human respiratory system consists of a series of organs and structures that are organized as shown in **Figure 1.5**. Although lungs represent the main respiratory components that control the air-breathing phenomenon, all the other biological structures of the respiratory tract are somehow involved in the air-breathing process (Maton et al., 2010).

2.1.2. Respiratory physiology

The respiratory system has mainly the breathing or ventilation function, but also immune, metabolic and endocrine functions. The ventilation is a complex process that gathers triggering nervous implication, inhalation and exhalation phenomena, which involve contraction and relaxation of the respiratory muscles, and gas exchanges. This ventilation process is controlled by the gaseous composition of arterial blood, the aortic and carotid structures, and the CO₂ and O₂ pulmonary sensors that govern the diameters of both pulmonary blood vessels and bronchioles (Lovelock, 1991; Tortora and Anagnostakos, 1987).

While the trachea serves as a filter to the air, its branches – main bronchi – represent the conduits that bring air to respective lungs.

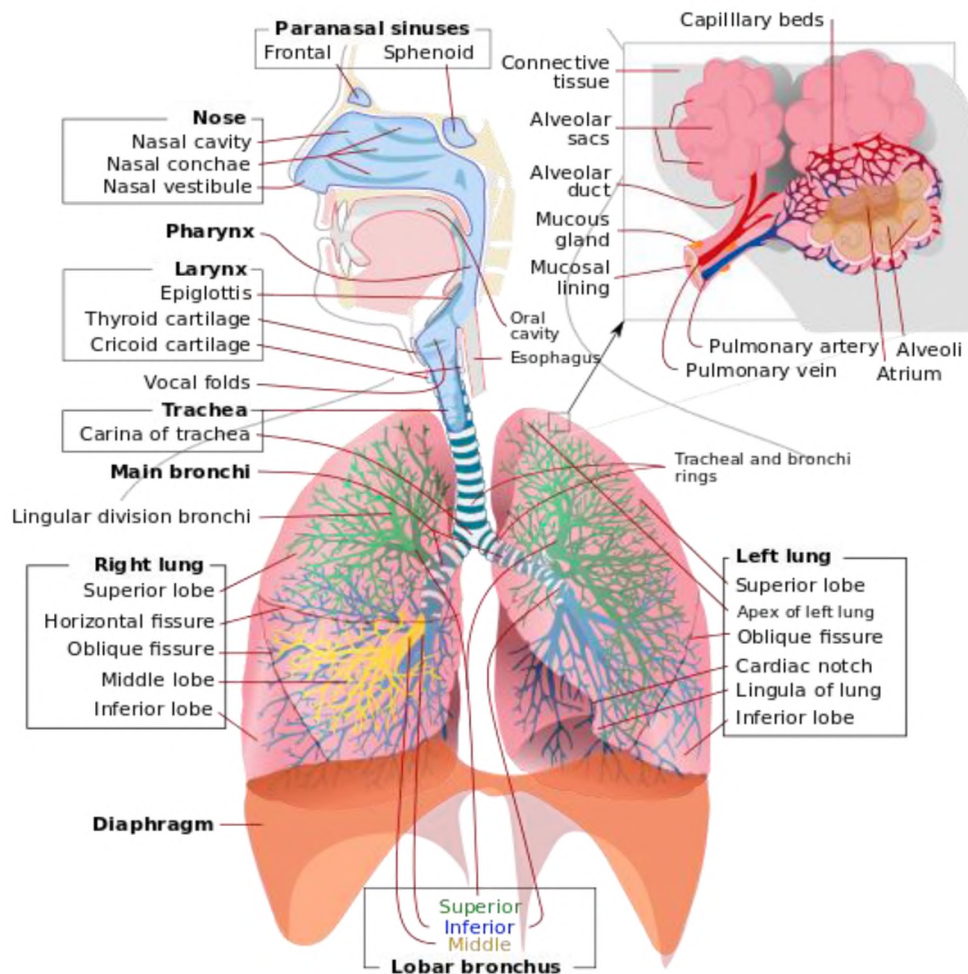


Figure 1.5: Comprehensive schematic anatomical representation of the respiratory system in humans. <https://www.mada.org.il/en/about/engineer/challenge/respiratory-system> (Accessed on October 30, 2016).

The surface of these air-conducting tubes is covered by cilia full of mucus that transports inhaled dust particles and microbes towards upper parts of the respiratory system for their elimination by coughing, sneezing, spitting or swallowing. This process, also called mucociliary clearance, is one of the major innate defense mechanisms that protect the lungs from biological inert, irritant and airborne particles (Kim, 2016; Maton et al., 2010).

2.1.3. Pathological consideration

The respiratory diseases comprise all the pathological conditions that affect the physiological or anatomic components of the respiratory tract. The disease conditions that affect the respiratory system can be categorized into chronic respiratory disease, restrictive lung diseases, respiratory tract infections, tumors, pleural cavity diseases, pulmonary vascular

disease and neonatal disease. The main characteristic of the chronic respiratory disease is the abundant presence of the inflammatory cells, namely nitrophiles, but also the devastation of the infectious agent in case of *Pseudomonas aeruginosa*. The most frequently encountered chronic respiratory diseases are asthma, bronchitis and emphysema that are characterized by a chronic pulmonary obstruction. In case of pediatric respiratory distress syndrome, poor lung compliance causes defective expansion and lack of lungs stiff that is characteristic to restrictive lung diseases (Thulasiramaraju et al., 2013).

The respiratory infections are classified into upper and lower respiratory infectious diseases (respectively URID and LRID) depending on the biological structure of the respiratory system that is affected. The most frequently occurring URID is the common cold; however, many other diseases affecting the upper part of the respiratory system, such as sinuses, pharynx, larynx, etc.; are part of the URID. On the other hand, the affection of the lungs, known as pneumonia, represents the most frequently encountered LRID. Among many other bacteria, viruses and fungus, *M. tuberculosis* is the most devastating infectious agents that cause pneumonia (Claire du Toit et al., 2006).

2.2. Inhalable drug delivery systems

2.2.1. Inhaled medications

As a route of administration, the respiratory tract is one of the oldest and has gained considerable attention. There are currently more than 65 clinically established inhalable pharmaceutical products based on more than 25 bioactive substances for lung diseases alone. In addition, several clinical trials for many other inhaled medications are ongoing (Thulasiramaraju et al., 2013; Labiris and Dolovich, 2003). A recent report by Clancy et al. (2013), for instance, has presented promising results of the clinical trial phase II for the efficacy and safety of inhalable amikacin-loaded liposomes; which have successfully reached the clinical trial phase III for lung infections (Pattni et al., 2015).

The anatomical and physiological organizations of the respiratory system represent the foundation of its biomedical applications, as a route of administration for local and systemic drug delivery. The lung is composed of about 300 million alveoli. Every alveolus is overlaid by capillaries giving a total of up to 280 pulmonary capillaries that represent approximately 70 m² of surface area as a blood–gas barrier. The thickness of this blood barrier, around 200–

500 nm, promotes the gas exchange and facilitates easy absorption of inhaled therapeutic materials (Kim, 2016; El-Sherbiny et al., 2015).

This high permeability along with the huge surface area represent the main characteristics of the lungs that render the systemic drug delivery possible and make the pulmonary route a great alternative to parenteral and oral administration. Pulmonary drug administration affords several other advantages over other routes: the avoidance of the first-pass metabolism, low pulmonary metabolic activity, non-invasiveness and possibility of self-administration. The pulmonary administration of anti-microbial agents offers high local bioavailability while reducing the side effects associated with oral or parenteral treatments (Cipolla et al, 2016a; El-Sherbiny et al., 2015; Van Rijt et al., 2014).

2.2.2. Challenges in pulmonary delivery

The respiratory tract has naturally developed some defense mechanisms that protect the lungs from airborne particles, which might be biological or inert foreign or toxic particles. The clearance mechanisms of the respiratory tract comprise i) the airway geometry, ii) mucociliary clearance, iii) alveolar macrophage uptake and iv) enzymatic degradation, each explained in some details below. While keeping unwanted inhaled particles away from the body, these defense mechanisms also usually affect inhaled medications and compromise the therapeutic effectiveness (El-Sherbiny et al., 2015; Van Rijt et al., 2014; Labiris and Dolovich, 2003).

Airway geometry and particle deposition. The onward branching and narrowing of the respiratory tract, from the trachea to the tertiary bronchi, contribute to the clearance function by preventing airborne particles from undergoing deep lung deposition. Like any other foreign airborne materials, inhaled therapeutic particles are also subjected to this phenomenon. The respiratory deposition of particles depends on several parameters such as the particle size and shape, flow of the incoming air, degree of bifurcation, the breathing rate and volume (Thulasiramaraju et al., 2013; Labiris and Dolovich, 2003). In inhalation, the size of particles is not considered as a geometric parameter, but an aerodynamic one. The aerodynamic particle diameter (Ad) is defined by the following equation (Owens *et al.*, 2002): $Ad = (\rho/F)^{0.5} \times d$. Where ρ is the density of the particle, F is the dynamic shape correction factor and d is the mass media particle diameter measured by optical microscopy.

The particle size represents the main factor that determines the region and mechanism of deposition. The most frequent mechanisms of deposition are impaction, sedimentation and diffusion. The airborne particles with sizes over 5 μm pass through the upper respiratory tract with high velocity and centrifugal force that make them to dash against the mucous respiratory wall and get deposited in oropharynx regions. This mechanism corresponds to the impaction that occurs mostly with the dry powder for inhalation and metered dose inhalators. When the particle sizes are in the range of 1–5 μm , the respiratory deposition occurs slowly in lower airways and bronchioles depending on the particle mass and the gravitational forces. This describes the mechanism of sedimentation that requires a slow breathing for the particles to settle and have long time span. Particles with sizes between 0.5–1 μm reach the deeper alveolar regions, where they are subjected to a rapid dispersion due to the presence of lung surfactants. This phenomenon promotes particle dissolution and diffusion in deeper respiratory areas. The respiratory diffusion also depends on the concentration gradient of inhaled particles (El-Sherbiny et al., 2015; Paranjpe and Müller-Goymann, 2014). Most of the particles with sizes less than 0.5 μm are exhaled, despite the fact that they also reach deeper lung regions. Despite their small sizes, inhaled therapeutic nanoparticles are reported to undergo deep lung deposition by a sedimentation process. This is explained by the aggregation of nanoparticles after their delivery from aerosolized preparations in the respiratory tract. These particulate aggregates are reported to be heavy enough for sedimentation and long life span in the lungs (El-Sherbiny et al., 2015; Paranjpe and Müller-Goymann, 2014).

Mucociliary clearance. This is the primary defense mechanism occurring in the upper respiratory regions. The surface of the airway is covered by cilia full of mucus produced by goblet cells that face the ciliated cells to form the mucociliary escalator system (MES). The ciliated cells are covered by a mucus layer and a periciliary layer. This latter layer furnishes the appropriate liquid environment that allows for the mobility of cilia during the clearance process, from the lung to the mouth. The MES captures airborne particles bigger than 6 μm and transports them towards upper parts of the respiratory system for their elimination by coughing, sneezing, spitting or swallowing (El-Sherbiny et al., 2015).

Alveolar macrophage uptake. Alveolar macrophages are part of the cells of mononuclear phagocytic system, which maintain the sterility in the lungs by clearance mechanism. Around 12–14 macrophages are located in each alveolus for this purpose. Inhalable particles with size range of 0.5–5 μm are generally subjected to alveolar macrophage uptake (Iida *et al.*, 2003),

while the optimum range is 1–3 μm (El-Sherbiny et al., 2015). This size-dependent clearance serves as a guide for the researchers dealing with pulmonary drug delivery. When the site of interest is beyond alveolar macrophages, the technology of inhalable particulate formulations controls the particles size in order to avoid this clearance mechanism. However, for some pulmonary infectious diseases like TB, this clearance mechanism is exploited as a therapeutic foundation for macrophage-targeting action using inhalable particles with suitable sizes (El-Sherbiny et al., 2015).

Enzymatic degradation. The enzymes belonging to the families of cytochrom P450 (CYP) play a primary role in the enzymatic degradation of inhaled therapeutic materials in the lungs. This process, also known as pulmonary detoxification, affects drugs of different structures, including theophylline, budesonide, salmeterol and ciclesonide. In addition, the presence of peptidases and proteases in the lungs limits also the bioavailability of peptide- and protein-based inhalable drugs. Though the metabolic activity occurring in other physiological systems is higher than that of the respiratory system, the enzymatic activity of the lungs deserves also some considerable attention in order to preserve the effectiveness of inhalable medications (El-Sherbiny et al., 2015). On the other hand, as with the need for macrophage targeting delivery, this local enzymatic activity represents an attractive area for the use of stimuli-responsive drug delivery systems.

2.2.3. Particulate systems for pulmonary delivery

The development of inhalable drug vehicles is one of the topics of current interest in drug delivery. Several inhalable particulate formulations are being studied for the management of both local and systemic diseases. In this technology, the anatomical and physiological characteristics of the respiratory tract are considered along with the properties of the drugs (El-Sherbiny et al., 2015). These factors allow for the formulation of effective inhalable particulate systems that overcome all the challenges of the respiratory route while controlling the release, improving the half-life, reducing the dosing frequency and the adverse effects of the therapeutic materials. The types of drug carriers used for pulmonary delivery are quite varied, from liposomes, solid lipid micro- and nano-particles, micelles, micro-emulsions, to polymeric micro- and nano-particles. **Table 1.2** presents typical particulate systems studied for pulmonary delivery of various therapeutic agents (adapted from Paranjpe and Müller-

Goymann, 2014). The schematic illustrations of the most commonly used inhalable nano-sized systems for pulmonary delivery of anti-microbial agents are presented in **Figure 1.6**.

Table 1.2: Particulate systems in pulmonary delivery of therapeutic agents for various applications (Paranjpe and Müller-Goymann, 2014).

Particulate systems	Therapeutic agents	Pulmonary applications
Liposomes	Budesonide Amikacin Anti-TB drugs Ciprofloxacin Surfactant agents Iloprost DNA/vaccine	Anti-inflammatory/anti-asthma Lung infections Lung infections Lung infections Surfactant therapy Pulmonary hypertension/heart failure Antibody and gene therapy
Solid lipid nano-/micro-particles	Budesonine Cucurmin Indomethacine Amikacin Anti-Tb drugs Tacrolimus Sildenafil Silibinin	Anti-inflammatory/anti-asthma Anti-inflammatory/anti-asthma Anti-inflammatory Lung infections Lung infections Lung infections Pulmonary hypertension/heart failure Anti-cancer
Polymeric nano-/micro-particles	Cucurmin Pirfenidone Methotrexate Paclitaxel Anti-TB drugs Moxifloxacin – Ofloxacin Tobramycin Clarithromycin Voriconazole – Itraconazole Sildenafil Carvedilol Small interfering RNA/gene Immunoglobiline Ig G1	Anti-inflammatory/anti-asthma Anti-inflammatory/anti-asthma Anti-cancer Anti-cancer Lung infections Lung infections Lung infections Lung infections Pulmonary hypertension/heart failure Pulmonary hypertension/heart failure Gene therapy Antibody delivery

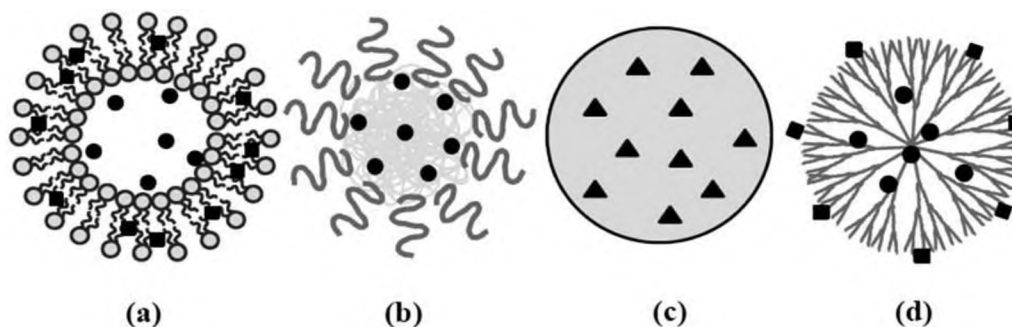


Figure 1.6: Schematic view of inhalable nano-systems for pulmonary delivery of anti-microbial drugs: liposome (a); polymeric nanoparticle (b); solid lipid nanoparticle (c) and dendrimer (d). The small black squares, balls and triangles represent various drugs encapsulated (from Zhang et al., 2010).

3. LIPOSOMES

Liposomes are lipid-based vesicular systems discovered and described in 1960 by Alec Bangham at the Babraham Institute in Cambridge, England. A few years later, the potential of liposomes for the delivery of pharmaceuticals, cosmetics and food ingredients was realised. Nowadays, liposomes represent attractive encapsulating devices that can improve the bioavailability and reduce the toxicity of current and emerging therapeutic compounds (Lila and Ishida, 2017; Bozzuto and Molinari, 2015). Due to the diversity of particle sizes, from 20 nanometers to several micrometers, liposomal vesicles are considered as either nanoparticles or microparticles. Liposomes can be made of natural or synthetic phospholipids organized into a supramolecular system having a bi-layer membrane that encloses aqueous medium. Liposomal vesicles are naturally endowed with both hydrophobic and hydrophilic regions that add more beneficial features in drug delivery (Bozzuto and Molinari, 2015; Pattni et al., 2015).

The advantages of liposomes as a device for the delivery of therapeutic agents include their biocompatibility, biodegradability, non-toxicity, non-immunogenicity, improved drug solubility and distribution, ability of performing co-delivery and the possibility of chemical modifications for targeting, controlled and sustained release. Although many promising results have been obtained with liposomes as drug vehicle, there are still some inconvenient sides in liposomal technology. These embrace the possible degradation of lipids by oxidation and hydrolysis, leakage, particles fusion and the costly formulation status due to the use of expensive lipids and sophisticated preparation methods (Li et al., 2015; Akbarzadeh et al., 2013). Another disadvantage of liposomes is their rapid uptake by the cells of mononuclear

phagocytic system (MNPS), which significantly decreases their half-life and limits their therapeutic application when the site of interest is beyond the immune cells. However, this longstanding issue in liposomal delivery is one of the most attractive features of this carrier for anti-microbial drugs, especially when the pathogen is located in the MNPS as it is the case with the mycobacterium (Lila and Ishida, 2017). On the other hand, a series of liposomes bearing polyethylene glycol (PEG) has been successfully developed and attractive therapeutic results have been obtained. These PEGylated liposomes, referred as stealth liposomes in comparison with conventional liposomes without PEG, can circulate longer in the blood stream and be useful for pharmacological purposes (Lila and Ishida, 2017). As shown in **Table 1.3**, many liposomal formulations are currently available on the market as registered pharmaceutical products for the treatment of various disease conditions.

Table 1.3: Liposomal products clinically approved for the treatment of some disease conditions (Bozzuto and Molinari, 2015; Pattni et al., 2015).

Marketed product	liposomal	Drug name	Therapeutic indications
Abelcet		Amphotericin B	Fungal infections
AmBisome		Amphotericin B	Fungal infections
Amphocil		Amphotericin B	Fungal infections
DaunoXome		Daunorubicin	Hematological malignancy
DepoCyt		Cytarabine	Lymphomatous meningitis
DepoDur		Morphine sulfate	Pain relief
Doxil		Doxorubicin	Kaposi's sarcoma and solid tumors
Epaxal		Inactivated hepatitis A virus	Hepatitis A
Evacet		Doxorubicin	Ovarian cancer
Inflexal V		Inactivated hemagglutinin of influenza virus strains A and B	Influenza
LipoDox		Doxorubicin	Kaposi's sarcoma and solid tumors
Marqibo		Vincristine sulfate	Acute lymphoblastic leukemia
Visudyne		Verteporfin	Photodynamic therapy

3.1. Formation

All liposomal systems derive from the dispersion of phospholipids in aqueous media. Phospholipids are famous amphiphilic compounds that have hydrophilic head groups and hydrophobic acyl chains (**Figure 1.7**). In the presence of water, phospholipids self-assemble and produce a bilayer structure. Within this system, all the polar head groups are lined up to form a water-attracting surface, while the hydrophobic tails of one lipid layer face the tails of the other layer producing a region away from water. This explains the encapsulation of multiple therapeutic agents, hydrophilic and hydrophobic drugs, in a single dosage form for mono or dual drug delivery. The schematic illustration of liposome formation is presented in **Scheme 1.2**. On the other hand, the use of amphiphilic compounds having a single hydrophobic chain produces a spherical system called micelle, which is formed due to the centripetal orientation of hydrophobic tails (Li et al., 2015; Kumar et al., 2012).

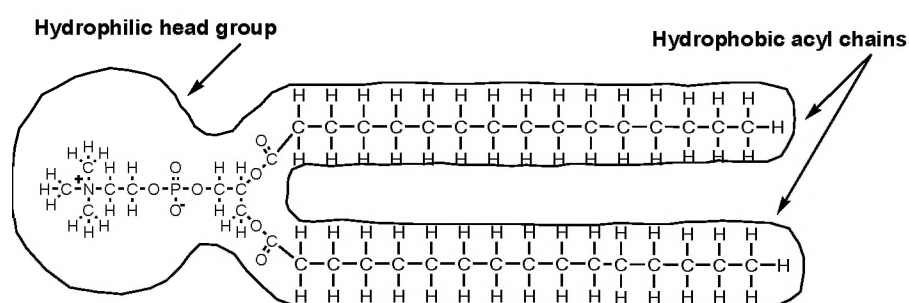
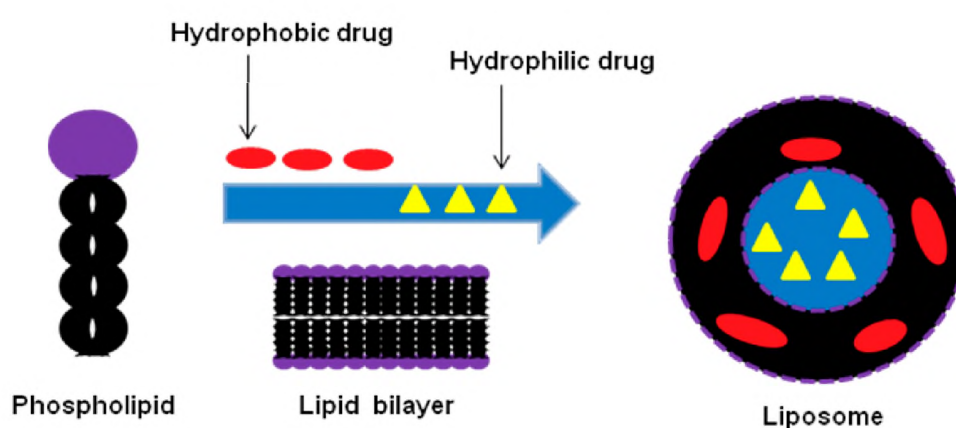


Figure 1.7: Illustrative view of the shape of phospholipid molecule (adapted from Kumar et al., 2012).



Scheme 1.2: Schematic illustration of liposome formation and drugs encapsulation.

3.2. Classification and Composition

3.2.1. Classification

Liposomes can be assigned to the following categories based on their lamellarity and size (Pattni et al., 2015; Tapaswi, 2013):

- Small unilamellar vehicles (SUV), size range 20 nm to 100 nm;
- Large unilamellar vesicles (LUV), size over 100 nm;
- Giant unilamellar vesicles (GULV), size over 1000 nm;
- Oligolamellar vehicles (OLV), size range 100 nm to 1000 nm;
- Multilamellar large vesicles (MLV), with size over 500 nm;
- Multivesicular vesicles, size from 1000 nm to several thousand nanometers.

According to the composition and functionalities, liposomes can be categorized as conventional liposomes, long circulating liposomes, cationic liposomes, stimuli–responsive liposomes and immunoliposomes. The differences between all these liposomes and the conventional liposomes are the following:

- long circulating liposomes are sterically stabilized liposomes that have been modified by grafting or coating a hydrophilic polymer, mostly PEG that increases the half–life of liposomes by decreasing their uptake by the cells of MNPS (Tapaswi, 2013);
- cationic liposomes are positively charged liposomes that are made of cationic lipids, which allow for encapsulation of genetic materials via electrostatic forces with the negatively charged phosphate backbones of nucleic acids (Paecharoenchai et al., 2012);
- stimuli–responsive liposomes are smart liposomal systems characterized by controlled releases of the encapsulated materials depending on a stimulus such as changes in pH, temperature, red–ox potentials, enzymes concentrations, ultrasound, electric or magnetic fields (Bozzuto and Molinari, 2015; Drummond et al., 2000);
- immunoliposomes are functionalized liposomes that have been subjected to surface modification by grafting antibodies for active targeting towards specific receptors at the site of interest (Kamboj et al., 2013; Tapaswi, 2013).

There are many other vesicular drug delivery systems considered as part of liposomal systems. These include emulsomes, enzymosomes, sphingosomes, transferosomes, ethosomes, pharmacosomes and virosomes that are lipid–based liposomes analogous. The non

lipid-based liposomes analogous are aquasomes, bilosomes and niosomes (Kamboj et al., 2013; Tapaswi, 2013).

Emulsomes are vesicular drug carriers made of high proportions of lecithin to stabilize a mixture of fats and triglycerides as an emulsion of oil in water to deliver hydrophobic drugs mostly by parenteral routes. Amphotericin B-loaded emulsomes have been developed for the treatment of visceral leishmaniasis (Kamboj et al., 2013).

Enzymosomes are vesicular supramolecular structures formed by complexation reaction between lipids and enzymatic proteins to be delivered. This strategy was used to deliver therapeutic enzymes such as Superoxide Dismutase for the management of oxidative stress (Kamboj et al., 2013).

Sphingosomes are vesicular systems made of sphingolipids in order to overcome the stability issues of liposomes due to hydrolysis or oxidation of phospholipids. Sphingolipids offer the advantage of containing amide or ether bonds that are more chemically stable than the ester bonds found in phospholipids. Vincristine-loaded sphingosomes have been successfully developed and clinically established as a registered pharmaceutical product called Marqibo® for the treatment of acute lymphoblastic leukemia (Pattni et al., 2015; Kamboj et al., 2013).

Transferosomes are malleable and hydrophilic lipid-based systems that are used for transdermal delivery. Due to their ability of squeezing and changing the shape, transferosomes have been successfully used for skin delivery of insulin and proteins of gap junctions (Tapaswi, 2013).

Ethosomes are phospholipid-based vesicular systems that contain diluted ethanol that increases significantly the encapsulation efficiency of hydrophobic drugs such as acyclovir, minoxidil and testosterone, and promotes transdermal and dermal delivery (Kamboj et al., 2013; Tapaswi, 2013).

Pharmacosomes are lipid-based vesicular systems formed by grafting covalently the therapeutic agent onto a phospholipid for liposome formation in order to overcome the issue of leakage. The pro-drug obtained shows attractive amphiphilic behavior that improves the bioavailability of hydrophobic drugs. Diclofenac-loaded pharmacosomes have enhanced the bioavailability and reduced the gastrointestinal toxicity of the trapped drug (Kamboj et al., 2013).

Virosomes are lipid-bilayered vesicular structures that contain viral glycoproteins, which allow for targeting the cells that are vulnerable to the virus of interest. Like viruses, virosomes are taken up by either membrane fusion with the cell or with endosomal membranes leading to cytosolic delivery of the encapsulated drugs (Kamboj et al., 2013).

Aquasomes are part of the most recent lipid-free vesicular systems made of a core based on tin oxide, diamonds (nanocrystalline carbon ceramic) or calcium phosphate dihydrate (brushite) that is coated with oligomeric film, which allows loading of the therapeutic agent by either copolymerization, diffusion or adsorption with or without modification (Kamboj et al., 2013).

Niosomes are vesicular drug delivery systems made of non-ionic surfactants, mostly spans and tweens. Unlike liposomes, niosomes are stable particulate drug carriers due to their phospholipid-free composition (Kamboj et al., 2013). Mukherjee et al. (2007) have reported higher stability of acyclovir-loaded niosomes and slower drug-release as compared to acyclovir-loaded liposomes made of soybean lecithin and cholesterol.

Bilosomes are niosome-like particulate delivery systems that contain bile salts, mostly deoxycholic acid, embedded in the membrane. This stabilizes bilosomes and prevents their gastrointestinal disruption by bile acids. The high absorption of bilosomes in small intestines makes these drug carriers appropriate devices for liver-targeting drug delivery due to the resulting hepato-circulation (Kamboj et al., 2013).

3.2.2. Composition

Liposomes are made of physiologically acceptable natural or synthetic phospholipids mostly found in the lipid bilayer membranes of human cells. The most frequently used phospholipids for liposomes preparation are phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS) and phosphatidylglycerols (PG). The molecular structures of these biocompatible lipids are shown in **Figure 1.8** and **Table 1.4** (Li et al., 2015; Pattni et al., 2015).

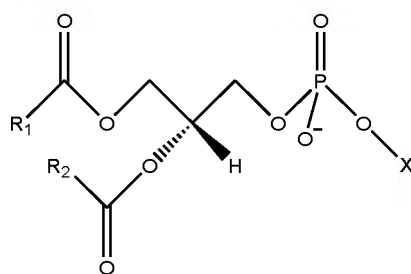


Figure 1.8: Structural representation of ester glycerol–phospholipid molecules, with R_1 and R_2 representing acyl chains of different fatty acids (adapted from Li et al., 2015).

Table 1.4: Description of –X moieties of different glycerol–phospholipids (adapted from Li et al., 2015).

Phospholipids name	PC	PE	PS	PG
—X Moiety				

In liposomal technology, considerable attention is given to the phase transition temperature (T_t) of these phospholipids. The T_t corresponds to the temperature above which phospholipids exist in liquid crystalline phase. In this fluid state, hydrophobic tails of phospholipids are randomly oriented but ready to form closely continuous bilayered structures – liposomes. As for the gel phase below the T_t , hydrophobic tails of phospholipids are completely expanded, well packed and thus not able to form liposomes (Pattni et al., 2015; Patel & Panda, 2012).

As most of the phospholipids used for liposomes formulation have T_t close to the physiological temperature (37 °C), the addition of cholesterol has been adopted as a strategy to stabilize the liposomal vesicles in physiological media. This is mostly for phospholipids that can undergo phase transition and leakage around physiological temperature, which can release the encapsulated drugs before reaching the site of interest. In fact, cholesterol strengthens the packing of phospholipid bilayered membranes and reduces their permeability as well as possible leakage. Some studies have also reported the ability of cholesterol to slow down drug release and increase the size of liposomes (Bozzuto and Molinari, 2015; Pattni et al., 2015).

Additive agents such as oleic acid and N-[1(2,3-dioleoyloxy) propyl]-N, N, N-trimethylammonium chloride (DOTAP) are useful for the preparation of negatively and positively charged liposomes, respectively. These charged liposomes offer the advantage of great liposomal stability during the storage, as charged particles repel each and reduce aggregation tendencies. While the cell internalization of positively charged liposomes is promoted by their electrostatic interaction with cell membranes that are negatively charged, liposomes bearing negative charges are subjective to poor cell internalization due to the corresponding repulsive forces. Cationic liposomes are used in gene therapy due to their ability to successfully encapsulate nucleic acids by electrostatic forces (Pattni et al., 2015; Paecharoenchai et al., 2012).

Cholesteryl hemisuccinate (CHEMS) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) are the most frequently used pH-sensitive lipids for the formulation of pH-responsive liposomes. CHEMS has the potential of expressing pH-sensitivity either alone or in the presence of other lipids. In ionized forms at basic or neutral pH, CHEMS stabilizes the lamellar form of DOPE in lipid-based vesicles. However, the protonated or molecular CHEMS formed at acidic pH promotes hexagonal phase of this lipid, which leads to the disruption of the vesicular systems and release of the encapsulated materials. Tocopherol hemisuccinate (THS) has also shown the same pH-responsiveness as CHEMS due to their molecular similarity (Xu et al., 2012; Xu et al., 2008; Hafez and Cullis, 2000).

3.3. Lipids profiles

In this study, crude lecithin from soybeans was used for liposomes preparation in company with cholesterol as a membrane stabilizer. The main reason behind the choice of this lecithin was its low cost that might be beneficial for the economical status of therapeutic liposomes.

3.3.1. Lecithin

Lecithin is a naturally occurring lipid mixture containing mainly phospholipids and glycolipids. The term lecithin is also sometimes referred to PC, which is the major phospholipid of crude lecithin. Lecithin derives either from animal source, specifically the yolk of eggs, or vegetable sources, rice bran oil or soybean oil. The vegetable lecithin (VL) is the most frequently used due to its high availability, accessibility and low cost. The VL also

offers the advantage of containing multiple physiologically acceptable and beneficial lipids, which correspond to the lipids found in the human cell membrane (Machado et al., 2014a).

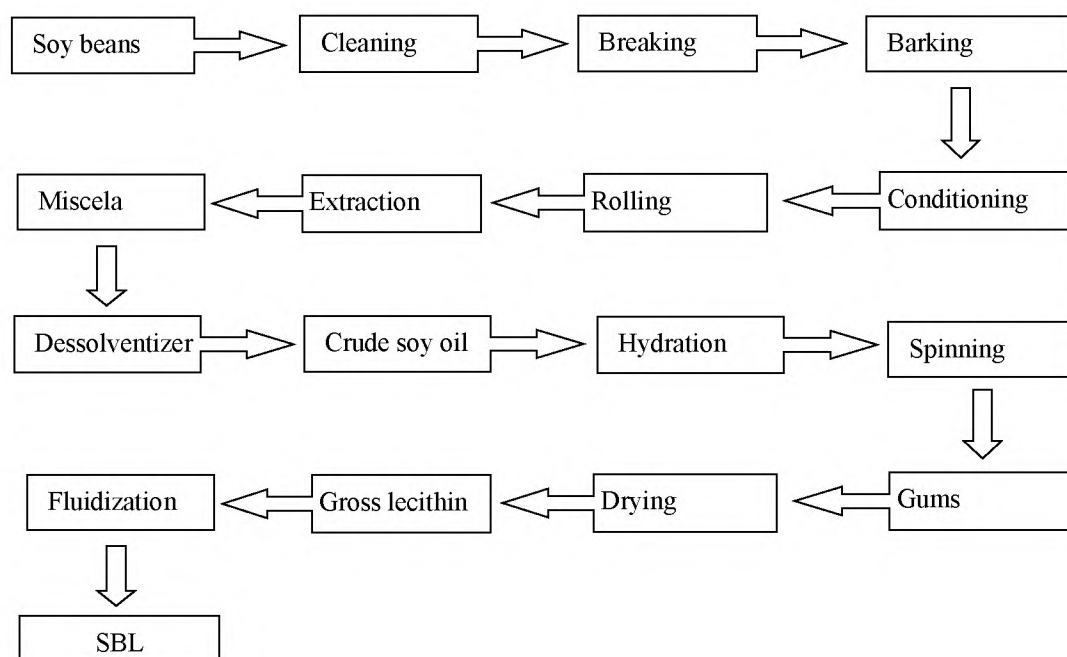
Soybean lecithin (SBL) is an internationally recognized safe food and pharmaceutical ingredient. The Food and Drug Administration (FDA) of the United States of America (USA) has approved the use of SBL in humans. The European Union has also accepted SBL and registered it under the number EE322. Nowadays, SBL is worldwide used as an emulsifier, lubricant and stabilizing agent for vitamins A and E in foodstuff, cosmetics and pharmaceutical industries. Due to the presence of PC, PE and PI; SBL is used also as a supplementary food to provide dietetic components such as choline and inositol (Machado et al., 2014a; Fernandes et al., 2012; Wu and Wang, 2004). SBL can be produced from soybeans oil following the procedure presented in **Scheme 1.3**.

3.3.2. Cholesterol

Molecular formula: $C_{27}H_{46}O$

Molecular weight: 386.67 g/mole

Structure: Cholesterol molecule consists of a bulky stereroid structure (four linked nuclei) that bears a hydroxyl group on one end and a hydrocarbon tail on the other end (**Figure 1.9**).



Scheme 1.3: Graphic illustration of the production of SBL from soybean (Machado et al., 2014a).

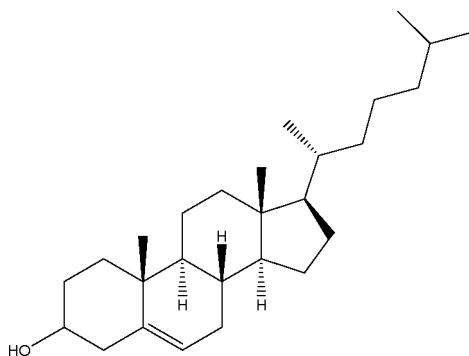


Figure 1.9: Molecular structure of cholesterol

Systematic name (IUPAC): (10R, 13R)-10, 13-dimethyl-17-(6-methylheptyl)-2, 3, 4, 7, 8, 9, 11, 12, 14, 15, 16, 17-dodecahycyclopenta[a]phenanthren-3-ol.

Melting point: 147 to 150 °C.

Cholesterol, also known as cholesteryl alcohol, is a white to yellowish lipid belonging to the lipid group of sterols that are part of many anatomical structures and have several physiological and biochemical functions. Cholesterol appears yellow to tan after a prolonged light and air exposure. The marketed cholesterol is mostly extracted from the wool fat or the spinal cord of cattle using petroleum ethers. The extracted material is subjected to a consecutive bromination for purification purpose. However, the synthesis of cholesterol through usual organic chemical reactions is also possible nowadays (Rowe et al., 2009; Carmichael, 2001).

Though cholesterol is known as a physiologically acceptable ingredient for pharmaceutical use, there are some experimental data discussing its teratogenic and effects on human reproduction (Sax, 2004). In addition, bovine spongiform encephalopathy related to the use of cholesterol obtained from animal species has been reported as a possible pathological manifestation. Chronic exposure to cholesterol may promote atherosclerosis and gallstones and cause eyes irritation. The thermal decomposition of cholesterol produces irritating fumes and acrid smokes. This possible toxicity suggests a particular care and safety consideration when handling cholesterol based samples (Rowe et al., 2009).

3.4. Preparation methods

3.4.1. Film hydration

The thin film hydration method (or Bangham method) is the simplest and oldest method used for liposomes preparation since 1961. This method involves drying the solution of lipid components in organic solvent and hydrating the resulting thin film with an appropriate aqueous solvent. The conditions of film hydration affect the structural organization of liposomal vesicles. While a forceful hydration of the lipid film produces multilamellar liposomes with heterogeneous sizes, a gentle hydration leads to the formation of giant unilamellar vesicles. Mostly, film hydration method requires an additional sizing treatment in order to produce liposomes with reduced sizes and confined distribution. This is most frequently done by probe and bath sonication techniques, which produce small unilamellar vesicles. The probe sonication is achieved by immersing a titanium-based nozzle in the liposomal suspension. The disadvantages of this method include the local hotness produced in the preparation that can affect the chemical stability of both lipids and the drugs, and the possible contamination of the formulation by titanium from the probe. The bath sonication is an effective alternative due to the possibility of controlling the sonication parameters while producing liposomes with equivalent structural properties. Another technique used for size reduction involves consecutive extrusion of the preparation through polycarbonate filters. The size reduction in this method is related to the number of extrusion cycles and the pore sizes of the filtering membranes. The main inconvenience of film hydration method is that the aqueous core to be entrapped is often limited. This results in poor encapsulation efficiencies of hydrophilic drugs (Pattni et al., 2015; Machado et al., 2014; Akbarzadeh et al., 2013).

3.4.2. Reverse phase evaporation

Reverse phase evaporation is an alternative method for liposomes preparation that is characterized by higher aqueous core loading as compared to the film hydration method. Initially, a water-in-oil emulsion is formed between the aqueous solution of the hydrophilic drug and the organic solution of lipids and any hydrophobic drugs. The organic solvent is removed at low pressure using rotary evaporator and the resulting film residue is redissolved in another organic solvent, mostly diethyl ether or isopropyl ether. Another portion of aqueous phase is added and the mixture is subjected to a short sonication that produces a homogeneous dispersion. A slow removal of organic solvent using the rotary evaporator leads to the

formation of a gel that is converted into a liposomal suspension. The liposomes are isolated from the liquid mixture by centrifugation, dialysis or sepharose 24 column. The main drawback of this method is about the persistence of the traces of organic solvents, which might be responsible of some stability and toxicity issues (Pattni et al., 2015; Machado et al., 2014a).

3.4.3. Solvent injection

This preparation method involves quick injection of the solution of lipids in either ethanol or diethyl ether in the aqueous medium maintained either at room temperature for the water–miscible solvent or at 60°C for the immiscible organic solvent, which gets evaporated in the process. Liposomes made during this injection process are often highly heterogeneous. The problems related to the use of solvent injection method include the difficulties associated with the removal of organic solvents, as ethanol forms azeotrope mixture with water, and the exposure of the therapeutic agents to be encapsulated to high temperature. The residues of organic solvents might also compromise the stability and the safety of the preparation (Pattni et al., 2015; Machado et al., 2014a).

3.4.4. Detergent removal

In detergent removal method, phospholipids are dissolved in aqueous solution containing detergents at critical micelle concentrations (CMC). The removal of the detergent releases phospholipids dispersed under molecular level, which allows for self-assembly into closely continuous lipid bilayered structures. The detergent can be removed by means of dialysis bag method. However, the most substantial removal of the detergent is achieved by the use of polystyrene-based absorber beads. The dilution of the medium using appropriate aqueous phase leads to the disorganization of micelle systems and release of phospholipid molecules that form liposomes. The detergent removal can also be accomplished by gel permeation chromatography using sulphadex columns. In this method, a pre-treatment of the polysaccharide beads with empty liposomes is required in order to saturate the beads with phospholipids and hence minimize the disruption of liposomes while traveling throughout the column (Pattni et al., 2015; Akbarzadeh et al., 2013).

3.4.5. Heating method

Heating method is a recent preparation technique that offers the advantage of not involving organic solvent in the process. In this method, liposomes components are hydrated using appropriate aqueous medium. The mixture obtained is heated above the transition temperature of the phospholipids used in the presence of glycerin 3% volume per volume. In the presence of cholesterol, the preparation is done over 120 °C due to the high melting point of this lipid. As this method involves heating process, the liposomes prepared can escape from any further sterilization process for perenteral use. Glycerin being a physiologically acceptable ingredient widely used in pharmaceutical fields, there is no worry about further removal of the remaining traces. In these liposomes, glycerin serves as an isotonicizing agent and stabilizer by preventing coagulation or sedimentation. This method also favors the freeze-drying process of liposomes due to the cryoprotective effect of glycerin. All these advantages make the heating method more simple and cost effective as compared to other methods. The application of this method is possible for both laboratory and industrial scales (Mozafari, 2005).

3.4.6. Microfluidic methods

The microfluidic methods gather all the novel preparation techniques that use micro-channels having diameters from 5 µm to 500 µm. The solution of lipids in ethanol or isopropanol is flowing perpendicularly or in opposite direction to the aqueous medium in the channels. An axial mixing of the two phases occurs continuously and liposomes are formed by local diffusion of phospholipid molecules that self-assemble in contact with the aqueous phase. The most frequently used technique in these methods is the micro hydrodynamic focusing, which leads to the production of mono dispersed small unilamellar and large unilamellar liposomes with size ranging from 40 to 140 nm. The other preparation technique includes the microfluidic droplets method that is employed to make giant liposomes in the size range of 4–20 µm, using hexane for phospholipid dissolution. Pulsed jet flow microfluidic technique is one of these recent preparation methods that produce giant liposomes with higher encapsulation efficiencies. The conventional film hydration has been adapted by drying the solution of lipids in microtubes, and hydrating the resulting lipid film by perfusion of the microtube that generates big vesicles, 200–534 µm (Pattni et al., 2015; Patil and Jadhav, 2014).

3.4.7. Supercritical fluidic method

The supercritical fluidic preparation method corresponds to reverse phase evaporation method that uses carbon dioxide (CO₂) maintained at supercritical temperature and pressure. In these conditions, CO₂ acts as a perfect solvent for the dissolution of lipids components for liposomes preparation. Using an HPLC pump, the aqueous dispersing medium is slowly added to a view cell containing the solution of lipids in CO₂ under supercritical conditions, which allow phospholipids to undergo phase transition. The decrease in pressure leads to a complete removal of CO₂ and self-assemble of phospholipid molecules into a continuously closed bilayered system. This preparation technique produces large unilamellar vesicles with size in the range of 0.1–1.2 µm and 5-fold higher encapsulation efficiency as compared to the conventional Bengham method. A part from being an organic solvent-free method, the supercritical fluidic method offers the advantage of using a cheap and environmentally harmless solvent, CO₂ (Pattni et al., 2015; Patil and Jadhav, 2014).

3.4.8. Drug loading

There are two main techniques to get the drugs entrapped into liposomes. These comprise passive loading and active loading methods. The encapsulation of therapeutic agents in liposomes by passive loading involves the treatment of hydrophobic drugs along with lipid components and the dissolution of the hydrophilic drugs in the aqueous dispersing medium. In the process of liposome formation, the water-soluble drug is spontaneously enclosed in the aqueous core of liposomes while the hydrophobic drug is embedded in the lipid bilayers. The encapsulation efficiency of hydrophilic drugs by passive loading is mostly limited, as the volume of the aqueous dispersing medium is often larger than that of the liposomal core. In general, the entrapment efficiency depends on the composition of liposomes – phospholipid types, phospholipid to cholesterol ratio and lipid to drug ratio – and the preparation method employed (Pattni et al., 2015; Akbarzadeh et al., 2013).

The active loading technique requires the creation of trans-membrane drug-concentration gradient, pH gradient or salts gradient, which results in rapid diffusion of drug molecules into liposomes through lipid membranes. Two of the marketed liposomal products, Doxil® and Myocet®, are prepared by active loading techniques using ammonium sulfate gradient and pH dependent gradient respectively (Pattni et al., 2015).

3.4.9. Freeze thawing

Freeze thawing technique is a post treatment of liposomes prepared that aims to improve the encapsulation efficiency and decrease the lamellarity and polydispersity of liposomes. This technique consists in freezing the preparation in liquid nitrogen and thawing afterward above the transition temperature of the phospholipids used. In this process, the lipid membranes become fluid and disrupted, allowing for vesicles fusion and substantial diffusion of drug molecules into the liposomes. Another possible explanation of increased encapsulation efficiency in this method is the cryo-concentration. This corresponds to the exclusion of drug molecules in concentrated regions during the freezing-thawing transitions. The number and duration of freeze-thawing cycles are crucial parameters to be controlled, as they can affect the encapsulation efficiency, lamellarity and polydispersity of liposomes (Costa et al., 2014; Sriwongsitanont and Ueno, 2011).

3.4.10. Freeze drying

The freeze-drying technique, also known as lyophilization, is a laboratory and industrial method commonly used to preserve the physicochemical properties of various products. Though organic co-solvent based solutions can also be subjected to freeze-drying treatment, the aqueous preparations are the most lyophilized products. This is because water molecules can promote any possible reactions that can speed up the decomposition of either active pharmaceutical ingredients or excipients in the formulation. Freeze-drying is useful especially for drying aqueous preparations containing thermally instable compounds, which might be degraded under heat-drying process. The principle of freeze-drying consists in freezing the samples and subsequently removing the resulting ice without water formation. This method has demonstrated attractive results regarding the improvement of the stability of liposomes under storage conditions. However, some studies have reported possible leakage of liposomes during the process of freeze-drying or suspension reconstitution. Recently, the addition of carbohydrates such as trehalose, mannitol, sucrose, etc; has been reported as a potential solution to overcome this stability issue of the freeze-dried liposomes. Among many others carbohydrates, trehalose was found to be the best cryo-protecting or freeze-protecting agent that guarantees the stability of liposomes during and after lyophilization (Akbarzadeh et al., 2013).

3.5. Characterization techniques

The physicochemical stability and biological fate of liposomes can be estimated by examining their characteristic properties. In general, liposomes are characterized by their size and polydispersity (or size distribution), zeta potential, shape and surface morphology, lamellarity, encapsulation efficiency, phase behavior or polymorphism, and *in vitro* release profile (Pattni et al., 2015; Prathyusha et al., 2013).

3.5.1. Size and shape analysis

The evaluation of liposome size is useful for biomedical applications and the quality control of liposomal batches. The analytical techniques used for the determination of liposome size include dynamic light scattering (DLS), size exclusion chromatography (SEC), field-flow fractionation (FFF) and microscope technologies including transmission electron microscopy (TEM), cryogenic-TEM (Cryo-TEM), and atomic force microscopy (AFM), which are also used to determine the shape and surface morphology (Bozzuto and Molinari, 2015; Pattni et al., 2015; Prathyusha et al., 2013).

DLS, also known as photon correlation spectroscopy or quasi-elastic light scattering, is the most frequently used technique for the evaluation of particle size and polydispersity. DLS evaluates the Brownian motion – that is the movement of particles due to their bombardment by solvent molecules – which leads to the scattering of the light applied. The instrument estimates the time-dependent fluctuation rate of the intensity of the scattered light, which is related to the diffusion speed of the dispersed particles. Smaller particles diffuse quicker than the bigger ones. The difference between the refractive index of the solvent and particles governs the degree of light scattering event. The average particle size is automatically calculated by estimating the amount of light that has been scattered. The measurement method is simple, fast and reliable for routine studies. DLS offers also the advantage of carrying out particle size analysis on a native liposomal preparation, and the size range to be evaluated is quite wide, from a few nanometers to several micrometers. Nevertheless, this technique is also limited to some extent. DLS does not differentiate individual particles from aggregates, and the effect of particle solvation cannot be subtracted from the readings recorded. The size measured by DLS is mostly considered as hydrodynamic diameters. Additionally, the particle size analysis by DLS is highly sensitive to particulate contaminants (Bozzuto and Molinari, 2015; Pattni et al., 2015; Hadinoto et al., 2013).

SEC is an analytical tool useful for the separation, quantification and size evaluation of liposomes. SEC resolves liposomes with various sizes by a classical fractionation and ensures their purification by the removal of non-vesicular impurities. The technique involves fast elution of big liposomes followed by smaller liposomal vesicles. The hyphenation of high performance liquid chromatography (HPLC) and SEC is a potential analytical strategy that separates and analyzes liposomes size with high resolution. However, false readings can occur as liposomes can squeeze in the pores or can be attached to hydrophobic components in the column packing and render the recovery tricky (Bozzuto and Molinari, 2015; Pattni et al., 2015; Grabielle-Madelmont et al., 2003; Lundahl et al., 1999).

FFF is an analytical technique that uses a discriminative membrane to allow only the dispersing liquid to pass through while returning liposomes. The laminar flow of the particles in the suspension and their size govern the separation by this technique. FFF operates high resolution of liposomes in a wide size range (Bozzuto and Molinari, 2015).

The microscopic observation of liposomes using TEM or Cryo-TEM instruments offers a precise reading of particle size. These techniques allow for visualization of liposomes as individual particles and provide their shape and morphology. In general, the liposomal dispersion is placed onto a copper grid and the solvent is allowed to dry before the analysis. The liposomes appear as black spherical particles on a white background. In negative staining TEM, the copper grid prepared is treated with a drop of the negative staining agent, uranyl acetate or phosphotungstic acid. This makes liposomes to appear as bright spherical spots on a black background. As TEM analyses liposomes out of their native environment, any changes in liposomal structure by shrinking, swelling or artifact formation is possible (Pattni et al., 2015; Chetanachan et al., 2008). Cryo-TEM is used in order to minimize liposome disruption by the sample preparation process. This technique relies on a flash freezing treatment and a controlled environment for the analysis. However, Cryo-TEM works perfectly only with small particles as compared to large ones. AFM has been recently developed to allow direct particle analysis from the native environment without any additional sample treatments. This technique offers also the advantage of high resolution of vesicle shape at three-dimensional level. Nevertheless, all these techniques require sophisticated expensive instruments that are not always available and accessible (Bozzuto and Molinari, 2015; Pattni et al., 2015).

3.5.2. Zeta potential

Zeta potential of liposomes corresponds to the sum of their surface charge, which depends on the lipid components used in the formulation. The analysis of liposomes charge is a useful tool used to estimate the stability during the storage and the biological fate. While highly negatively or positively charged liposomes do not aggregate significantly in the preparation, neutral and slightly charged liposomes tend to form huge aggregates due to the lack of effective repulsive forces. To measure the zeta potential, DLS estimates the fluctuations in the intensity of the scattered light due to the mobility of liposomes. This is caused by the impact of the electric field applied on particle charges. The charge on the surface of liposomes governs their mobility that creates a change in the intensity of the light scattered (Pattni et al., 2015; Hadinoto et al., 2013).

3.5.3. Encapsulation Efficiency

The evaluation of encapsulation efficiency (EE) requires the separation of drug-loaded liposomes from the free drug. This can be achieved by means of centrifugation, dialysis or column separation. After this separation, the EE can be evaluated by direct quantification of the encapsulated drug. This requires a previous disruption of drug-loaded liposomes using one of the following chemical products: acetonitrile, ethanol, methanol or Triton X-100. On the other hand, the indirect estimation of EE is done by analyzing the free drug isolated and considering the initial drug amount engaged in the formulation. According to the physicochemical properties of the drugs, various analytical techniques can be used for quantitative analysis, such as ultraviolet (UV) spectroscopy, fluorescence spectroscopy, enzyme- or protein-based assays, gel electrophoresis, HPLC, UPLC or LC-MS (Pattni et al., 2015).

3.5.4. Lamellarity

The lamellarity of liposomes is part of their structural characteristics that can have an impact on the intended applications. The number of lipid bilayers can be evaluated using chemically labeled or radiolabeled agents that can be distributed in the bilayer membranes. However, this technique is limited due to the fact that these reagents might be distributed only on the outer lipid membrane and lead to false readings. To date, Cryo-TEM is the most commonly used technique for the determination of lamellarity by visualization (Pattni et al., 2015). The nuclear magnetic resonance spectroscopy of the 31-phosphorus (^{31}P -NMR) is also being

currently used to estimate the lamellarity of liposomes. This technique deals with the estimation of the ratio of phospholipid amount in the outer layers to that of the inner layers (Frohlich et al., 2001). The ^{31}P -NMR spectrum with a broad peak indicates the presence of MLV while a narrow peak corresponds to SUV. The addition of paramagnetic ions such as Mn^{2+} , Co^{2+} , and Pr^{3+} shifts the respective peaks to either downfield or upfield as a result of ionic interactions with the phosphate backbone. By comparing the spectroscopic profile with and without the paramagnetic ion, the lamellarity of liposomes can be estimated. Some other techniques such as small-angle X-ray scattering (SAXS) and trapped volume determination can be used to estimate liposome lamellarity (Pattni et al., 2015; Bouwstra et al., 1993).

3.5.5. Phase behavior

The most frequently used techniques for investigating the phase behavior of liposomes include thermal analysis techniques, especially thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC), and X-ray diffraction (XRD). By examining the thermal behavior and crystallinity, these techniques provide valuable complementary information for characterization and quality control of the liposomal formulations (Singh et al., 2015; Omwoyo et al., 2014; Prathyusha et al., 2013).

TGA consists in monitoring the mass of the sample as a function of temperature or time while it is subjected to a programmed heating, cooling or isothermal treatment in a controlled atmosphere. The instrument used is referred as a thermobalance or thermogravimetric analyzer. The outcome of TGA experiment corresponds to a mass loss curve or TGA curve that describes the profile of mass changes against temperature. A derivative curve, called DTG for derivative thermogravimetry, is often drawn in order to clearly describe the decomposition steps recorded. The maximal curvature of DTG curve corresponds to an inflexion of TGA curve that occurs when the weight loss is faster (Finkelstein, 2005; Giron, 2002).

DSC measures the difference in electric power (heat flow) between a sample and a reference – which are both subjected to a programmed heating, cooling or isothermal treatment in a controlled atmosphere – as a function of temperature or time. The technique that deals with the difference in temperature instead of heat flow is called differential thermal analysis (DTA), which is an equivalent technique to DSC. In DSC, the sample pan and empty reference pan are heated either by the same heater, heat flux DSC, or by separate heaters, power compensated DSC. The pans used in DSC experiments are based on aluminium, tin,

zinc and/or indium. When the sample undergoes any phase transition, such as fusion or crystallization, the electric power is adjusted in order to maintain the temperature in the sample as equivalent to the reference. Throughout the experiment, the amount of electric power required is converted into a plot of heat capacity against temperature or time. The analysis of this DSC curve, also called heat flow curve, provides the respective transition temperature and enthalpy and allows for the determination of the nature of thermal events. While the DTA curve gives downward melting peaks, the endothermic peaks in DSC are oriented upward as the sample absorbs an amount of heat in order to melt. The exothermic peaks in DSC curve are oriented normally downward. However, in some instrumental technology, the two thermal events are reversed following DTA curves (Giron, 2002; Ohline et al., 2001; Koyama et al., 1999; Haines, 1995). DSC is the most commonly used thermal analysis technique in the characterization of lipid-based materials in terms of polymorphism, glass transition, melting and crystallization behavior, amorphous and crystalline states (Singh et al., 2015; Omwoyo et al., 2014; Prathyusha et al., 2013; Castelli et al., 2005; Ohline et al., 2001; Koyama et al., 1999).

Unlike the techniques for thermal analysis that do not allow samples recovery, XRD is a non-destructive analytical technique used to evaluate the crystalline and amorphous states of the materials. This technique takes the advantage of the ability of X-rays to penetrate the material and allow for the collection of information under atomic structure level. The instrument is an environmentally and user-friendly device that is easy to use. A wide range of materials such as powders, solids and liquids can be quickly assessed by XRD. In addition, this technique is characterized by high resolution, reliability and cost effectiveness in terms of cheap maintenance, easy data collection, processing and interpretation. XRD is highly sensitive to impurity and can be useful for the evaluation of the purity of materials with crystal structures. XRD allows the study of phase transitions and assesses the degree of polymorphism and solvatomorphism. XRD is widely used in pharmaceutical and chemical industries for the development, production and quality control of the inert (excipients) and biologically active materials. In nanotechnology, XRD is largely used to determine the size of nanoparticles that are characterized by crystalline structures, such as inorganic nanoparticles based on nickel and iron ions (Chauhan A and Chauhan P, 2014; Dorofeev et al., 2012; Kirtansinh et al., 2011).

3.5.6. *In vitro* drug release

The evaluation of release profile of the drug from liposomes is mostly done by dialysis bag method. The liposomal preparation is trapped into a dialysis bag of specific molecular weight cut off. The obtained tubing membrane is placed in a receptacle medium, which is often a simulated buffered physiological liquid, maintained under a controlled stirring and temperature depending on the intended application. Following a well-designed timing schedule, a specific volume of the release medium is withdrawn for drug analysis and an equivalent volume of the fresh buffer is replaced. The drug is quantified using an appropriate analytical method, such as UV spectroscopy, fluorescence spectroscopy, enzyme- or protein-based assays, gel electrophoresis, HPLC, UPLC, LC-MS, etc. The release profile is estimated by plotting the percentage released against the time intervals chosen, zero order release model (Pattni et al., 2015). Many other release models can be used to analyze the mechanism of drug release. These embrace plotting the logarithm of the percentage of drug remaining against time, first order model; the percentage of drug released versus the square root of time, Higuchi's model; and the logarithm of percentage released against the logarithm of time, Korsmeyer-Peppas plots (Sipemmann and Peppas, 2001).

3.6. Applications

3.6.1. Applications in drug delivery

Overview of liposomal drug delivery. Extensive studies conducted in liposomal technology have led to the development of several effective liposomal formulations that are currently used in clinics (**Table I.3**). Nowadays, liposomes represent the most clinically appointed drug vehicle for human diseases (Bozzuto and Molinari, 2015; Guptha A, 2015). From this successful use, liposomes have gained considerable attention in biomedical fields. Liposomes are being studied for the delivery of existing and emerging drugs at various research levels, from basic stages related to research and development to preclinical and clinical trials. The use of liposomes in drug delivery aims to modify the pharmacokinetics of drugs in order to improve the therapeutic efficacy while minimizing associated adverse effects (Akbarzadeh et al., 2013).

Liposomal formulations are being addressed to skin related diseases, cancer therapy or infectious diseases via dermal or trans-dermal, oral, pulmonary or parenteral administrations (Pattni et al., 2015). Cancer therapy is known to be a potential field for the application of

liposomes due to poor bioavailability and side effects of anti-cancer drugs. However, some common infectious diseases – such as tuberculosis, leishmaniasis and fungal infections – seem to be also interesting candidates for the use of conventional liposomes that are effectively engulfed by the cells of MNPS (Lila and Ishida, 2017).

Apart from the nature and localization of the disease, the design and development of liposomes depend also on the intended administration route – as different anatomical and physiological characteristics can be encountered from one route to another (Prathyusha et al., 2013). Liposomes have also been studied for brain targeted delivery in order to treat Parkinson's and Alzheimer's diseases. Small liposomes have shown promising results due their ability of crossing the brain blood barrier by simple diffusion or mannose mediated internalization for mannosylated liposomes (Pattni et al., 2015; Prathyusha et al., 2013).

Liposomal pulmonary delivery. The advantages of liposomal systems in drug delivery, such as uniform local deposition, enhanced bioavailability and reduced side effects; have attracted the attention of researchers in the field of pulmonary delivery. In addition, the composition of liposomal systems, especially the lipid components that are chemically similar to lung surfactants, makes liposomes appropriate drug carriers for pulmonary delivery. This is due to the lung surfactant mimic action that enhances the biocompatibility and reduces any possible irritating or toxic effects of therapeutic materials (Paranjpe and Müller-Goymann, 2014). Liposomal preparations for inhalation can be presented either in liquid or solid state depending on the needs. The lipid composition, particle size and charge, lipid to drug ratio and delivery method represent the main parameters that determine the deposition and effectiveness of liposomes in the respiratory tract. Therefore, the objectives behind pulmonary liposomal delivery should govern all the settings for the preparation of desired liposomes (Bai et al, 2009). The first inhalable liposomal preparation to be approved for the clinical application was Alveofact®, which is based on purified bovine surfactant and used to treat pediatric acute respiratory distress syndrome. Currently, two more inhalable liposomal formulations, ciprofloxacin-loaded liposomes (Pulmaquin®) and Amikacin-loaded liposomes (Arikace®), are being studied in clinical trials for the treatment of respiratory infectious diseases. As illustrated in **Table 1.2**, many other studies are being conducted for pulmonary liposomal delivery of drugs and various other therapeutic agents such as genetic and anti-oxidants substances (Paranjpe and Müller-Goymann, 2014).

3.6.2. Application in gene delivery

Liposomes have been reported to achieve effective intracellular delivery of genes. These liposomes are made of cationic lipids, which allow for encapsulation of genetic materials via electrostatic forces with the negatively charged phosphate backbones of nucleic acids. The positive charge on the surface of liposomes also influences their interaction with negatively charged cell membranes and promotes cell internalization (Paecharoenchai et al., 2012). Cationic liposomes offer several advantages over viral gene vectors, including the easy and safe production, cost effectiveness, possibility of monitoring toxicity, biodegradability, biocompatibility, and lack of dangerous immunogenicity. However, liposomes are suffering from poor transfection efficiency due to their limited endosomal escaping ability, which exposes the genetic materials to enzymatic and acid degradation in lysosomes. This has been improved by incorporating fusogenic lipids, such as DOPE, in the liposomal formulation. These particular lipids facilitate endosomal escaping by membrane fusion with endosomes, leading to early cytoplasmic release of the gene. Though cationic liposomes have also shown some dose-dependent toxicity, successful results have been obtained in cancer therapy when delivering genes encoding for tumor suppression proteins. The liposomal systems for targeted gene delivery to epithelial cells of the respiratory tract have also demonstrated promising results for the treatment of cystic fibrosis (Dizaj et al., 2014; Haritha et al., 2012).

3.6.3. Application in vaccine delivery

The use of specific lipids or molecules such as phosphatidylserine, DOTAP, fatty acids and monophosphoryl lipids can produce liposomes with attractive immune-stimulating activities. Liposomes carrying antigenic materials, either encapsulated in the aqueous core, grafted or coated on the surface; are capable of stimulating immune responses after their macrophage uptake. Following the endolysosomal degradation, macrophages present the antigen to T-lymphocytes that initiate the production of cytokinines and specific antibodies via activation of B-lymphocytes (Pattni et al., 2015). The immune response produced by liposomes can be influenced by their composition, lamellarity, size and surface charge (Hussain et al., 2014). Liposomes containing trehalose 6,6'-dibehenate and a cationic lipid, dimethyldioctadecylammonium bromide, in a 1: 5 mass ratio have demonstrated efficient delivery of the TB vaccine Ag85B-ESAT-6. This vaccine has shown prolonged immune response without any toxic effects. However, the replacement of this cationic lipid by neutral lipids led to a significance decrease in immune response (Dissel et al., 2014). While the

liposomal vaccine targeting the major histocompatibility class I complex (Stimuvax) for lung carcinoma was not successful, two liposomal vaccines have been clinically established for the treatment of hepatitis A and influenza virus infections. These vaccines are known as Epaxal and Inflexal V respectively (**Table 1.3**). Both of the formulations are based on virosomes, which offer the advantage of undergoing membrane fusion either with the cell or the endosomes leading to efficient cytosolic delivery (Bozzuto and Molinari, 2015; Pattni et al., 2015; Kamboj et al., 2013).

3.6.4. Application in diagnosis

The use of liposomes for diagnosis purposes is one of the topics of current interest in biomedical applications. Liposomes with magnetic properties, also called magneto-liposomes, are made by entrapping superparamagnetic iron-based nanoparticles or iron oxides or gadolinium (III) chelates for magnetic resonance imaging. The encapsulation of quantum dots and fluorescent dyes into liposomes has also led to the development of attractive liposomal platforms for diagnosis. Additionally, liposomes have shown great potential for simultaneous accommodation of drugs and diagnostic agents, such as radionuclides, magnetic or contrast substances. This can be achieved by encapsulation in the inner core, embedding in the lipid bilayer, chemically grafting or coating onto the surface of liposomes. A successful targeted co-delivery of these materials has given to liposomes the status of theranostic systems, as they provide both the therapeutic effect and the diagnostic control (Lila and Ishida, 2017; Bozzuto and Molinari, 2015).

3.6.5. Application in food encapsulation

As versatile lipid-based systems, liposomes have shown some potential in the encapsulation of food ingredients. In this field, the use of liposomes aims to stabilize some nutraceutical or dietetic ingredients during the storage, to improve their organoleptic characters or to provide a controlled and targeted delivery of these substances in a specific tissue. The shelf lives of highly instable bioactive products such as vitamins, enzymes and anti-oxidative agents have been prolonged by their encapsulation in liposomes (Machado et al., 2014a). Yokota and collaborators (2012) have successfully improved the bitter taste and ugly odor of casein hydrolysate in dietetic preparations by liposomal encapsulation. In addition to the encapsulation of dietetic compounds, liposomes have been recently used to encapsulate a

cyanobacterium, namely *Spirulina platensis*, as a proteins source. The liposomes prepared demonstrated high encapsulation efficiency and attractive uniform particles morphology (Machado et al., 2014b).

3.6.6. Application in analytical fields

In analytical domains, liposomes can be usefully involved in immunoassays, biosensors analysis and liquid chromatography. Liposomes can be used to encapsulate, embed or conjugate the analytical entities with high loading capacity and huge surface area that can enhance the intensity of the analytical signal. In a direct enzyme-linked immunosorbent assay (ELISA) like method, fluorophore-conjugated liposomes carrying a substantial amount of secondary antibody quickly bind to the antigen that has been fixed on the primary antibody. The addition of a colorimetric substrate leads to color development and allows for analytical estimation of the antigenic analyte. The use of liposomes has been deemed to lower the limits of detection of analytes and increase the sensitivity of the immunological analysis technique (Pattni et al., 2015; Singh et al., 1996). In chromatography, the conjugation of liposomes to the stationary phase for gel permeation chromatography is useful for the separation of drugs and proteins. This is also useful for the study of possible molecular interactions on the phospholipid membranes. Additionally, the cell-like appearance of liposomes makes them appropriate simulated cell models for the study and prediction of the interactions between biologically active compounds and cell membranes (Pattni et al., 2015).

4. REPORTS ON ANTI-TB DRUG DELIVERY AND LIPOSOMES

- Recently, Bhardwaj et al. (2016) formulated isoniazid-ciprofloxacin-loaded pH-responsive liposomes containing 4-aminophenyl- α -D mannopyranoside as macrophage targeting agent. Cholesteryl hemisuccinate (CHEMS) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were used as pH-sensitive agents and liposomes were made by film hydration preparation method. The release profile evaluated at different pH media over 24 hours showed a fast-kinetic release of the drugs in macrophage acidic conditions, up to 81–87%; while the percentage release in neutral and alkaline media was around 58–64%. *In vitro*, pH-sensitive liposomes decorated with ligand demonstrated higher alveolar macrophage uptake as compared to non-decorated liposomes. The pulmonary delivery of the liposomes

evaluated *in vivo* showed maximum concentration of the drugs co-delivered in the lungs. Overall, the results of this study demonstrated the possibility of improving the bioavailability of anti-tubercular drugs using pH-sensitive macrophage targeting liposomes.

- Singh and co-workers (2015) have developed rifampicin-loaded lipospheres containing phospholipids in company with sulfphobutyl ether β -cyclodextrin and Vitamin C. The formulation was characterized using various techniques such as differential scanning calorimetry, X-ray diffraction, Fourier transform infrared and cascade impactor. The proposed formulation demonstrated good flow ability, aerodynamic diameter 1.76–3.99 μm , and *in vitro* anti-tubercular activity. The emitted dose and fine particle fraction, evaluated as 80–93% and 68.84–83.73% respectively, were attractive for pulmonary delivery. Overall, the properties of this formulation revealed sound promising for effective control of tuberculosis by pulmonary administration route.
- Patil et al. (2015) have designed rifampicin-loaded liposomes by thin film hydration method followed by freeze-drying technique to produce a respirable liposomal preparation. These liposomes were characterized by dynamic light scattering, scanning electron microscopy, optical microscopy, differential scanning calorimetry, Anderson cascade impactor and *in vitro* anti-tubercular activity. The liposomal formulation containing soy lecithin and cholesterol in a 3:2 mass ratio and drug to lecithin in 1:5 mass ratio was deemed the best based on encapsulation efficiency, 79.25%, and controlled release behavior. This freeze-dried liposomal preparation was evaluated *in vivo* by intra-tracheal instillation and oral administration to the Wister rat models. With a controlled and sustained release profile, the optimized liposomal formulation demonstrated much better pharmacokinetics than the free drug.
- Rifampicin-loaded inhalable liposomes were formulated using dry film hydration method. The liposomal formulations were composed of either natural soy phosphatidylcholine or its hydrogenated derivative in company with cholesterol or oleic acid. Dynamic light scattering, transmission electron microscopy, phase analysis light scattering and rheological behavior were used for evaluation purpose. The selected drug-loaded liposomes were those made of natural soy phosphatidylcholine and cholesterol in a 3:1 mass ratio. This liposomal preparation showed the average particle size 112 ± 4 nm, zeta potential -57 ± 6.6 mV and encapsulation efficiency $74 \pm 4\%$. The addition of cholesterol led to an increase in particle size while the presence of oleic acid decreased the viscosity of the developed inhalable

formulation. In addition, the selected rifampicin-loaded inhalable liposomes demonstrated good cellular up take with less toxic effect toward cells of alveolar epithelium, as compared to liposomes containing oleic acid that dramatically reduced cell viability (Manca et al., 2012).

- Vyas et al. (2004) have evaluated the macrophage-targeting potential of inhalable rifampicin-loaded liposomes based on egg phosphatidylcholine and cholesterol in a 7:3 mass ratio. On one hand, these liposomes were decorated with maleylated bovine serum albumin and O-steroyl amylopectin as specific receptor targeting ligands and, on the other hand, dicetylphosphate was incorporated as a negatively charged agent. The ligand-anchored and negatively charged liposomal aerosols demonstrated enhanced drug concentration in alveolar macrophages over a prolonged period. And these liposomes have showed also higher clearance rate of the *Mycobacterium smegmatis* residing in rat macrophages, 89–93% versus 54.3% and 68.4% given by the pure drug and non-decorated neutral liposomes. This explains the preferential accumulation of modified liposomes in the macrophages. Based on the percentage of inhaled preparations to reach the deep lung, the aerosol performance of the formulations packed in a pressurized system using chlorofluorocarbon as propellant were studied. The obtained results were 1.5–1.8 times higher than what obtained with the ordinary aerosol of pure rifampicin solution.
- To produce a dual therapeutic system for tuberculosis treatment, pulmonary medication and lung surfactant mimic action; inhalable isoniazid-loaded liposomes were formulated by thin film hydration method using dipalmitoyl phosphatidylcholine (DPPC). The obtained liposomal formulation was evaluated in terms of particle size, encapsulation efficiency, drug release profile, deep lung deposition, antimicrobial efficacy and biocompatibility. The prepared drug-loaded liposomes showed an average size around 750 nm, about 36.7% of isoniazid encapsulation efficiency and up to 27% of deep lung deposition, as determined by impingement chamber. These liposomes could deliver more than the effective dose of the free isoniazid (above the minimum inhibitory concentration). DPPC-based isoniazid-loaded liposomes have demonstrated a lung surfactant mimic action, probably due to the fact that DPPC is the main lipid component of the lung surfactant. This might be useful in improving pathological conditions related to the depletion of endogenous lung surfactant due to internal or external causes such as the presence of *Mycobacterium tuberculosis* in alveoli. Moreover, the absence of cholesterol as lipid helper was justified by its possible inhibitory effect on the potential of DPPC as lung surfactant (Chimote and Banerjee, 2010).

- Rojanarat et al. (2011) have developed proliposomal dry powder formulations for pulmonary delivery of isoniazid (INH). These inhalable INH-loaded proliposomes were prepared by spray drying formulation technique using mannitol as bulking agent. Differential scanning calorimetry, X-ray diffraction and cascade impactor were used to characterize the formulations developed. *In vitro* antimicrobial efficacy towards *Mycobacterium bovis* located in alveolar macrophages and the cytotoxicity using the respiratory cell lines were evaluated. The encapsulation efficiency of INH in proliposomes was in the range of 18–30%. The formulations did not stimulate alveolar macrophages to produce inflammatory mediators. Overall, the inhalable INH-loaded proliposomes have shown attractive flow ability, powder performance and biological effects that are promising for TB therapy.

- Patil-Gadhe and co-workers (2014) have prepared and evaluated *in vitro* antimycobacterial activity and cytotoxicity as well as *in vivo* toxicity of inhalable rifapentine-loaded proliposomes. The results have demonstrated that the formulated proliposomal preparation could show high antimicrobial efficacy at 10µg/ml of rifapentine while no cytotoxicity could be found in A549 cells. *In vivo* studies in Wistar rats showed that rifapentine-loaded proliposomes formulation was safe from 1 to 5 mg of rifapentine/kg, but the doses from 10 mg/kg could be fatal for the animals.

- Rifampicin–isoniazid–loaded multilamellar liposomal vesicles made of phosphatidylcholine and cholesterol in a 2:1.5 mass ratio demonstrated a remarkable sustained drug release in alveolar macrophages by pulmonary administration to guinea pigs. While the pure drugs could not last over one day in the plasma, findings showed that a single nebulization of the prepared liposomal formulation offered an effective plasmatic drug concentration standing up to two days. The encapsulated drugs could be recovered in the lung five days after the application of nebulization (Pandey et al., 2004).

- Gürsoy et al. (2004) have encapsulated simultaneously rifampicin (RIF) and isoniazid (INH) in liposomes prepared by film hydration method followed by ultra-filtration to control the particle sizes. These liposomes were composed of egg-yolk phosphatidylcholine type XI-E (EPC) and dipalmitoylphosphatidylcholine (DPPC) in company with cholesterol (Chol) in a 2:1 molar ratio. The two drugs were loaded according to their solubility in the phospholipid

to drug molar ratio of 1:0.024 for RIF and 1:145.5 for INH, which correspond to the respective mass ratio of 0.000027:1 and 0.027:1 in the case of DPPC. This indicates that the mass ratio of RIF to INH in the liposomes was 1:1000. The derivative UV spectroscopic method was established as analytical technique for simultaneous quantification of the drugs in liposomes. The respective encapsulation efficiencies (EE) of DPPC-based and EPC-based liposomes were RIF $81.53 \pm 2.06\%$ and INH $73.84 \pm 0.78\%$, and RIF $74.45 \pm 0.48\%$ and INH $64.61 \pm 0.51\%$. Both the higher EEs and controlled release profiles of the dual liposomal system were more attractive than RIF-loaded liposomes and INH-loaded liposomes prepared separately.

- Justo and Moraes (2003) have attempted to co-encapsulate five anti-TB drugs in a single inhalable liposomal dosage form using a mixture of phosphatidylcholine and cholesterol in a molar ratio of 6:4. Though isoniazid and pyrazinamide were successfully loaded (with vesicle diameters varying from 286 to 329 nm), the encapsulation of rifampicin, ethionamide and streptomycin was not substantial. However, the development of multiple delivery systems for TB therapy remains a considerable idea, as this approach is in close agreement with the current multi-drug therapy for TB. The simultaneous liposomal delivery of anti-TB agents would offer an effective and safe medication while limiting the development of drug resistant strains.
- A review by Pattni et al. (2015) has presented huge background information on liposomes manufacture, focusing on the lately published preparation and characterization methods with a slight mention on the current applications. The conditions for liposomes formation, drug encapsulation and drug release study were pointed out along with sterilization approaches of liposomal preparations. The dry film hydration, reverse phase evaporation and solvent injection methods were highlighted as traditional methods along with the most recently developed techniques, namely micro hydrodynamic focusing, supercritical reverse phase evaporation and supercritical anti-solvent methods. As compared to the firstly cited conventional manufacture techniques, which involve easy set up for experimental level, the novel methods offer some possibilities for industrial scale production. However, these methods require sophisticated equipments that make them to be more demanding. In addition, the authors have provided a general outline of various common approaches to study different characteristics of liposomes in order to estimate both the shelf life and half-life. In terms of applications, this review has reported most of the clinically established

liposomal formulations and those under clinical trials, with special mentions regarding the potential of inhalable liposomes for pulmonary diseases like tuberculosis.

➤ Machado and collaborators have published a recent review highlighting the feasibility of using crude soy and rice lecithin for liposomal encapsulation of food ingredients. The review presented important features and notions related to soy and rice lecithin, and the preparation and characterization techniques of liposomes as well as their current applications. The collected data showed that the naturally occurring phospholipid mixture, both crude and purified lecithin from either soy or rice, could be useful for liposomes preparations regardless the expected fields of application. The possibility of using this cost-effective lipid mixture, non-purified lecithin, in liposomal formulations for medical, pharmaceutical or foodstuff purposes was discussed in this review paper as an attractive strategy to deal with the economical status of liposomes, as costly encapsulating devices (Machado et al., 2014a).

5. PROJECT JUSTIFICATION AND OBJECTIVES

5.1. Project justification

The main issues in the management of TB embrace the limited bioavailability of some anti-TB drugs, mostly due to the first pass metabolism along with nonselective bio-distribution, and high drug load needed for combination therapy. These result in a lengthy treatment with several adverse effects causing poor patient compliance. All these factors often lead to the therapeutic failure and promote the development of drug resistant strains (Manca et al., 2012; Sosnik et al., 2010), which explain the deadly status of TB and justify the urgent need for new curative strategies.

Considering the biological localization of *Mycobacterium tuberculosis*, mostly inside of alveolar macrophages (Tăbăran and Catoi, 2014), the use of inhalable drug vehicles that can be engulfed by the cells of mononuclear phagocytic system would be a promising therapeutic approach. The liposomes, which represent the most clinically appointed drug carriers (Guptha, 2015), would be appropriate potential candidate in this regard, as these are known to be avidly taken up by the immune cells (Bozzuto and Molinari, 2015; Pattni et al., 2015).

However, the costly formulation status of liposomes, due to the use of expensive synthetic or purified natural phospholipids (Li et al., 2015 and Yokota et al., 2012), is a limitation to the treatment of a poverty related infectious diseases like TB.

5.2. Project objectives

The aim of this work was to develop liposomes for pulmonary delivery of anti-tubercular drugs using crude soybean lecithin, which is an FDA-approved naturally occurring emulsifier that is quite cheap and readily available to produce cost-effective liposomal formulations.

The specific objectives of this study include:

- To design and develop liposomes for isoniazid encapsulation using crude lecithin;
- To validate the HPLC method for isoniazid (INH) quantification;
- To prepare and evaluate INH-loaded liposomes;
- To incorporate RIF as a hydrophobic model drug in order to obtain dual liposomes;
- To validate the HPLC method for simultaneous quantification of INH and RIF;
- To characterize the dual drug-loaded liposomes.

CHAPTER TWO

FORMULATION OF ISONIAZID LOADED LIPOSOMES

A part of this work was orally presented during the 2016 All Africa Congress on Pharmacology and Pharmacy in Muldersdrift and the 2016 Frank Warren Conference at Rhodes University.

A manuscript is under preparation for submission to the International Journal of Pharmaceutics.

1. INTRODUCTION

Liposomes represent versatile colloidal systems that are useful for various applications, including biomedical, dietetic and cosmetic fields. The settings for the design and development of liposomes are established based on the intended purpose, which mostly requires specific characteristics. The size and surface charge of liposomes have been identified as crucial factors to be controlled for effective pulmonary delivery of therapeutic materials (El-Sherbiny et al., 2015; Prathyusha et al., 2013; Nagayama et al., 2007). Isoniazid (INH) is a first-line anti-TB agent that has been reported to be poorly encapsulated in liposomes. This had been correlated to both the well-known limitations of liposomal systems regarding the encapsulation of hydrophilic compounds and the small size and hydrophilicity of INH (Pattni et al.; 2015; Rojanarat et al., 2011).

The main objective of this chapter was to formulate liposomes with high encapsulation efficiency for INH and desired properties for macrophage-targeting pulmonary delivery. This chapter aimed also to evaluate the feasibility of using crude soybean lecithin to encapsulate INH as a model drug. This was done in order to look at any opening towards the development of cost-effective liposomes for drug delivery. The following graphical abstract (**Figure 2.1**) illustrates briefly how the formulation of INH-loaded liposomes was achieved.

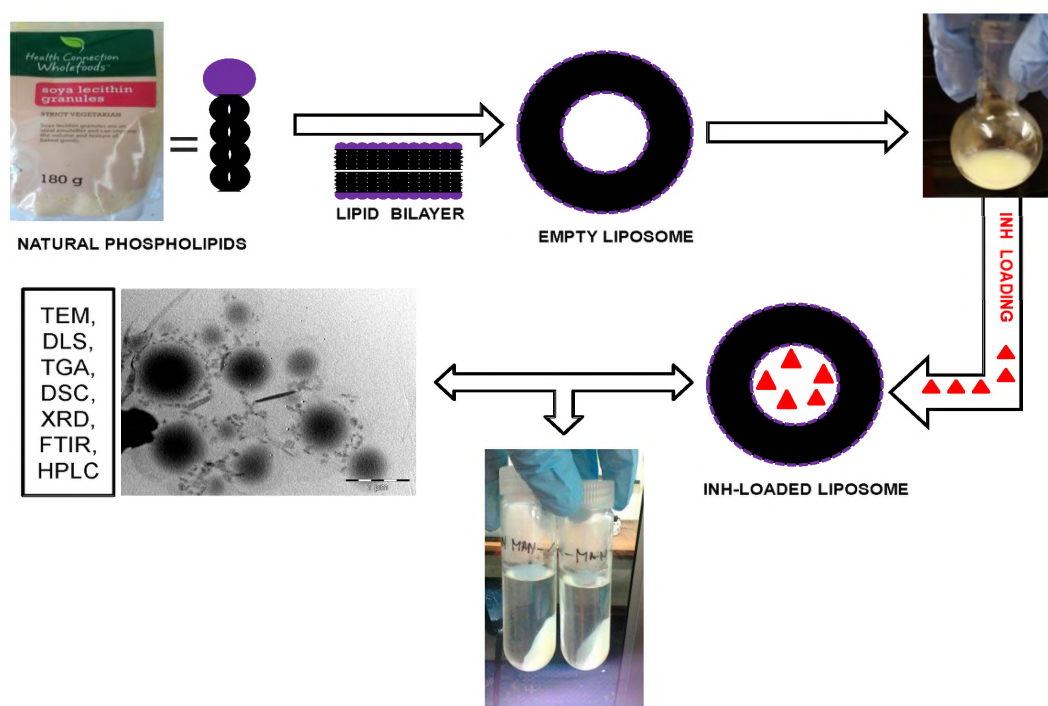


Figure 2.1: Flowchart illustrating the formulation of INH-loaded liposomes using crude soybean lecithin.

2. EXPERIMENTAL SECTION

2.1. Materials

Soybean lecithin granules used in this study were from Health Connection Wholefoods (USA). According to the manufacturer, these granules (100 g) contained mainly phosphatidylcholines (23 g), phosphatidylinositols (14 g), polyunsaturated fats (35 g), saturated fats (13 g), glycaemic carbohydrates (8 g), sodium (0.11 g) and total energy up to 2,940 kJ. Cholesterol used was from Carlo Erba / Divisione Chimica (Italy). Isoniazid, mono- and dibasic sodium phosphate were purchased from Sigma Aldrich (Germany). Ethyl acetate and acetone used were from Protea Chemicals (South Africa), while chloroform was from BM Scientific / Parow Industria (South Africa). Methanol from Merck (Germany) and acetonitrile from Ranbaxy Fine Chemicals Ltd (India) were of analytical high performance liquid chromatography (HPLC) grade. Unless indicated, all the chemicals were used as purchased without further purifications.

2.2. Methods

2.2.1. Preformulation studies

2.2.1.1. Drug–excipients compatibility

The evaluation of the physicochemical compatibility between the active pharmaceutical ingredient (API) and excipients is a highly recommended approach for preformulation studies. This preliminary assessment is done in order to guarantee the stability of the API in the dosage form and avoid any physicochemical incompatibilities that might compromise the bioavailability and efficacy of the drug (Patel et al., 2003; Mura et al., 1998).

INH was physically mixed with crude soybean lecithin and cholesterol in the mass ratio of 1:1 to make up a total mass of 20–30 mg. The physicochemical stability of INH was evaluated using some techniques commonly employed for solid state analysis of pharmaceutical ingredients. These include DSC, XRD and FTIR (Finkelstein et al., 2005; El-Hagrasy et al., 2001; Pifferi et al., 1999).

DSC. The phase behavior of the physical mixtures was evaluated in comparison with pure INH using PerkinElmer DSC-6000 instrument that gives upward endothermic peaks. The sample (3 mg) was heated from 30 to 450 degrees celcius (°C) at a flow rate of 10 °C/min in a nitrogen saturated atmosphere. The inert nitrogen gas flow was set at 20 ml/min. The

reference was an empty aluminum-based pan. The thermograms with respective heat flow curves were recorded and data were processed using the software DSC Pyris manager series.

XRD. The crystallinity of the physical mixture prepared was evaluated using Cu-K α radiation set at 1.5404 angstrom with a nickel filter, on Bruker D8 Discover instrument equipped with a Lynx Eye detector. The samples were placed on a glass sample holder and the diffraction patterns were recorded in the range of 2θ from 10 to 60 ° with a step size of 1 °/min and a slit width of 6.0 mm.

FTIR. The intactness of the functional groups that are characteristic to INH was assessed in the physical mixtures by FTIR, which is a potential analytical tool that evaluates the vibration frequencies of chemical bonds (El-Hagrasy et al., 2001; Silverstein, 1998). The FTIR spectra were recorded by the attenuated total reflection method using PerkinElmer Spectrum 100 FTIR Spectrometer. For each experiment, 8 scans were done in the frequency range of 650 to 4000 cm⁻¹ and data were processed using the software FTIR Spectrum.

2.2.1.2. Preparation and evaluation of empty liposomes

2.2.1.2.1. Preparation of empty liposomes

This section dealt with the screening and selection of some process and formulation parameters in order to produce liposomes with desired properties by film hydration method. Two series of liposomal batches were prepared and evaluated on the basis of particle sizes, size distribution (polydispersity index, PDI) and zeta potential. For each preparation series, three parameters that could affect the output variables were selected at different levels as shown in **Table 2.1**. The software for general statistics Minitab (version 12) was used for full factorial design of experiments to generate 27 possible combinations corresponding each to a liposomal formulation. The evaluation of the first series liposomes (S1) allowed to define some particular parameters for the preparation of the second series of batches (S2). Based on the evaluation parameters established, one of the second series liposomal formulations was selected for drug loading study.

Table 2.1: Preparation parameters selected for the first series (S1) and second of liposomes batches (S2).

Series	Parameters	Levels		
		1	2	3
S1	Lecithin-cholesterol mass ratio	1 : 0	3 : 1	2 : 1
	lipid-aqueous phase mass ratio	1 : 10	1 : 20	1 : 30
	Sonication time (minutes)	15	30	45
S2	Hydration time (min)	30	60	90
	Vortex time (min)	0	5	10
	Sonication time (min)	0	10	20

The film hydration procedure described by Lugo & Ghaly (2015) and Machado et al. (2014b) was used for liposome formulation with various modifications according to the preparation parameters under investigation.

Briefly, 100 mg of lipid components was dissolved in 1 ml of chloroform in a clean 25 ml round bottom flask. The obtained organic solution was dried at 60 °C using a rotary evaporator (Büchi Rotavapor R-205, Switzerland) set at 200 rotations per minute (rpm) for 5 minutes (min). The round bottom flask was removed and stored in a desiccator overnight at room temperature. Depending on the formulation, 1 to 3 ml of deionized water was added and the thin lipid film was allowed to hydrate at 60 °C under stirring at 400 rpm for a specific time period. The preparation was then homogenized by vortexing (Deluxe Vortex Mixer, Chiltern MT19) for 5 min or as indicated and the bath sonicator (Digital Ultrasonic Cleaner, spellbound 909) was used accordingly at 60°C. The obtained yellow suspension was stored at 4°C for further analysis.

2.2.1.2.2. Evaluation of empty liposomes

The liposomal batches were evaluated by dynamic light scattering (DLS) to determine the particle size, PDI and zeta potential. The morphology of particles and their individuality in the suspensions were revealed by transmission electron microscopy (TEM). The liposomal

formulation selected in the preparations series 2 was subjected to a quick stability study looking at any change in zeta potential values over 3 weeks.

Particle size and zeta potential. The liposomal dispersions were diluted to 1/5 using deionized water to reduce the opalescence. Zetasizer nano ZEN-3600 MAL1043132 (Malvern Instruments) was used at a scattering angle of 173 °. The measurements were performed in triplicate at 25 °C using either an ordinary cuvette for particle size determination or a capillary cuvette for zeta potential measurements.

Transmission electron microscopy. A drop of the liposomal preparation from DLS experiment was deposited on the copper grid and the liquid in excess was absorbed using a piece of filter paper. The samples were allowed to dry overnight beforehand and the microscopic observation was done on a Zeiss Libra – 120KV TEM instrument.

2.2.1.3. Validation studies for isoniazid quantification

Validation studies were conducted in order to ensure that the HPLC method for INH quantification was suitable under actual analytical conditions. The most widely applied validation parameters, namely the linearity range, accuracy, repeatability (intraday precision) and intermediate precision (inter-day precision); were evaluated consecutively.

The chromatographic system was consisted of an Agilent HP1100 LC-MSD and equipped with a quaternary pump, degasser, DAD detector, 1100 MSD and ChemStation for collection and analysis of data. ZORBAX Elipse Plus C18 4.6 x 150 mm x 5 micro column was used for reverse phase HPLC analysis. The mixture of aqueous solution of monobasic sodium phosphate 0.01M and acetonitrile (90:10) was used as mobile phase on isocratic elution mode. From a 1000 µg/ml stock solution of standard INH in Millipore water, five different concentrations (5–500 µg/ml) were prepared and filtered using 0.45 µm Millipore filters before their injection.

Over six days of experiments, three daily injections of 20 µl for each INH fresh solution were done onto the HPLC instrument. The flow rate of the mobile phase was 1 ml/min and the UV detection was set at 254 nm. The duration of each elution was 5 minutes and the retention time for INH was approximately 1.2 min. The average peak areas obtained were plotted against the respective concentrations to construct the calibration curve in order to determine the linearity range, regression equation and correlation coefficient. The percentage recovery

and relative standard deviations (RSD) were calculated in order to evaluate accuracy, repeatability and intermediate precision. The limit of detection (LOD) and limit of quantification (LOQ) were established as the ratio of the standard deviation for the lower concentration in the calibration curve to the slope of the regression equation times 3 for LOD and 10 for LOQ (ICH, 2005 and CLSI, 2004).

2.2.2. Preparation of INH-loaded liposomes

2.2.2.1. Purification of crude lecithin

For comparison purpose, purified lecithin was produced from crude soybean lecithin following the procedure described by Mertins et al. (2008). Briefly, the crude soybean lecithin (10 g) was dissolved in ethyl acetate (100 ml). Millipore water (4 ml) was added while stirring slowly in order to prevent the formation of two phases. After allowing the liquid mixture to stand, the supernatant was decanted off and the bottom phase, which looked like a gel, was dispersed in acetone (60 ml). The agglomerates formed were destroyed using a glass rod. Thereafter, acetone was decanted off and a further portion of acetone (60 ml) was added, repeating the crushing process. Finally, the precipitate was filtered under vacuum and allowed to dry in a desiccator connected to a vacuum pump for 48 hours. The purified lecithin (7.99 g) was collected and stored at room temperature.

2.2.2.2. Preparation of drug-free liposomes

Drug-free liposomes were prepared following the preparation conditions selected during the preformulation studies. In brief, 75 mg of crude lecithin (or purified lecithin) and 25 mg of cholesterol were dissolved in 1 ml of chloroform in a clean 25 ml round bottom flask. The obtained organic solution was allowed to dry at 60 °C using rotary evaporator set at 200 rpm for 5 min. The round bottom flask was removed and stored in a desiccator overnight at room temperature. Three ml of phosphate buffer (pH 7.0) in Millipore water was added to the thin lipid film and the mixture was heated for 60 min at 60 °C under stirring at 400 rpm. The mixture was then homogenized in a bath sonicator at 60 °C for 20 min to produce a yellow liposomal suspension. For comparison purpose, 3 ml of phosphate buffer (pH 7.0) was treated in the same conditions to produce a control solution free from lipid.

2.2.2.3. Encapsulation of isoniazid

Briefly, the hot yellow suspension of drug-free liposomes obtained above (section 2.2.2.2) was directly added to the aqueous solution of isoniazid (INH), made by dissolving 100 mg, 200 mg or 400 mg of INH, respectively, in 1 ml of phosphate buffer (pH 7.0) in the Beckman centrifuge tube. After being gently shaken, the mixture was subjected to 2, 4 or 6 cycles of freeze-thawing process according to Costa et al. (2014). For each freeze-thaw cycle, the centrifuge tube containing the preparation was immersed in liquid nitrogen (−196 °C) for 5 min and then in oil bath set at 65 °C for 10 min. Thereafter, the mixture was allowed to cool at room temperature for 60 min and 20 ml of Millipore water was added to produce a volume level for centrifugation. The positive control, lipid-free solution previously prepared, was treated in the same way while INH was not added to the drug-free liposomes (empty liposomes) used as negative controls.

2.2.2.4. Determination of encapsulation efficiency

The above liposomal mixture (section 2.2.2.3) was centrifuged at 20,000 rpm for 20 min using Beckman Coulter Allegra 64R centrifuge at room temperature. The supernatant was decanted off and the remaining liposomes were rinsed five times with 20 ml of Millipore water to remove the free INH molecules. These isolated liposomes were freeze-dried (Lyo Lab 3000, Apollo Scientific cc) for further evaluations. INH in the supernatant and in the positive control solution was quantitatively determined using the validated HPLC method. Various dilutions up to 1/100 and filtration of the sample with 0.45µm Millipore filters were done prior to the HPLC analysis. The quantitative analysis was done in triplicate and, for each replicate, three injections of 20 µl were done into the HPLC instrument according to the method described in section 2.2.1.3. To calculate the encapsulation efficiency (EE), the concentration of INH in the supernatant was subtracted from the INH positive control concentration used as a theoretical concentration and the percentage EE (%EE) was estimated according to the equation (2.1) shown below (Consta et al., 2014; Singh et al., 2011). Based on the %EE determined, only liposomes made of crude lecithin were subjected to a comprehensive characterization further.

$$\%EE = \frac{\text{Control concentration} - \text{Supernatant concentration}}{\text{Control concentration}} \times 100 \quad (2.1)$$

2.2.3. Characterization of INH-loaded liposomes

2.2.3.1. Determination of particle size and zeta potential

The freeze-dried preparations were gently dispersed in de-ionized water at room temperature to produce a homogeneous suspension. The measurements for particle size, size distribution and zeta potential were performed using the procedure and instrumentation described in preformulation studies (section 2.2.1.2.2).

2.2.3.2. Particle shape analysis

The aqueous liposomal dispersions from particle size and zeta potential analyses above were used for TEM experiments. The microscopic observation was conducted using the procedure and instrumentation described in section 2.2.1.2.2.

2.2.3.3. Thermal analysis

2.2.3.3.1. Thermogravimetric analysis

Thermogravimetric analysis (TGA) was used in order to study the thermal behavior of INH-loaded liposomes made of crude lecithin (CL) in comparison with their equivalent empty liposomes and pure INH. About 3 mg of each sample was heated in nitrogen-saturated atmosphere using PerkinElmer TGA-4000 instrument. The heat range was from 30 to 800 °C at a flow rate of 10 °C/min. The inert nitrogen gas flow was set at 20 ml/min. TGA thermograms with respective mass loss curves were recorded and data were analyzed using the software TGA Pyris series.

2.2.3.3.2. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used for the study of the polymorphism of INH-loaded liposomes made of CL in comparison with their equivalent empty liposomes and pure INH. The experiment was conducted using the procedure and instrumentation described in the section related to the drug-excipients compatibility study (section 2.2.1.1).

2.2.3.4. X-Ray Diffraction

XRD patterns were established in order to assess the crystalline state of INH in CL-based liposomes in comparison with the pure drug and empty liposomes. The experiment was conducted using the procedure and instrumentation described in section 2.2.1.1.

2.2.3.5. Fourier transform infrared

The presence and intactness of the functional groups of INH in freeze-dried CL-based liposomes were investigated by Fourier transform infrared (FTIR) spectroscopy. The IR spectra were recorded as described in the section that evaluates drug–excipients compatibility (section 2.2.1.1).

2.2.3.6. Drug release study

INH-loaded liposomes were firstly evaluated for drug content using the procedure described by Rojanarat et al. (2011) with a slight variation regarding aliquots. The sample (5 mg) was placed in a 25 ml volumetric flask containing methanol (10 ml) to destroy liposomal structure. The obtained dispersion was topped to volume using Millipore water. The volumetric flask was placed in a bath sonicator set at 35 °C for 30 min to promote drug dissolution, and the obtained solution was analyzed by the validated HPLC method. The experiment was performed in triplicate and three injections were done into the HPLC instrument for each replicate. The content of the drug (%DC) was estimated using the following equation:

$$\%DC = \frac{\text{Amount of drug recovered}}{\text{Amount of the formulation used}} \times 100 \quad (2.2)$$

The release profile of INH-loaded liposomes was determined according to the slightly modified procedure used by Chimote and Banerjee (2010) for pulmonary delivery. Briefly, an amount of liposomes corresponding to 5 mg of INH was gently dispersed in 5 ml of phosphate buffer (pH 7.4) in a 10 ml volumetric flask at room temperature. The volume was topped to 10 ml using the same dispersing medium. One ml of the prepared suspension was transferred into the dialysis tubing cellulose membrane (Membra-Cel MD 10-14 x 100 CLR, Sigma Aldrich). The dialysis bag was placed in a size-10 glass vial containing 20 ml of the phosphate buffer. The content of the vial was maintained at 37 °C under a stirring condition of 100 rpm. After 0.5; 1; 1.5; 2; 3; 4; 5; 7; 9 and 12 hours, 5 ml of the draining medium was withdrawn for INH quantification using the validated HPLC method. An equivalent volume of fresh buffer was added to the release medium for its renewal after every sampling. The free drug was treated in the same conditions and all the experiments were done in triplicate. Four release models (zero order, first order, Higuchi and Korsmeyer-Peppas) were evaluated and the model with the highest coefficient of determination (R^2) was considered for allocation of the release mechanism (Costa and Lobo, 2001).

3. RESULTS AND DISCUSSION

3.1. Preformulation studies

3.1.1. Drug–excipients compatibility

DSC. The DSC thermograms of mixtures of INH with crude lecithin (CL) and cholesterol are presented in **Figure 2.2**. The melting peak of pure INH appears at around 173.1 °C, which is in between 170–174 °C reported in the literature (BP, 2013). Though a slight decrease in melting point can be observed in the DSC curves of the physical mixtures, the presence of this endothermic peak suggests there is no major physical change in the structure of INH.

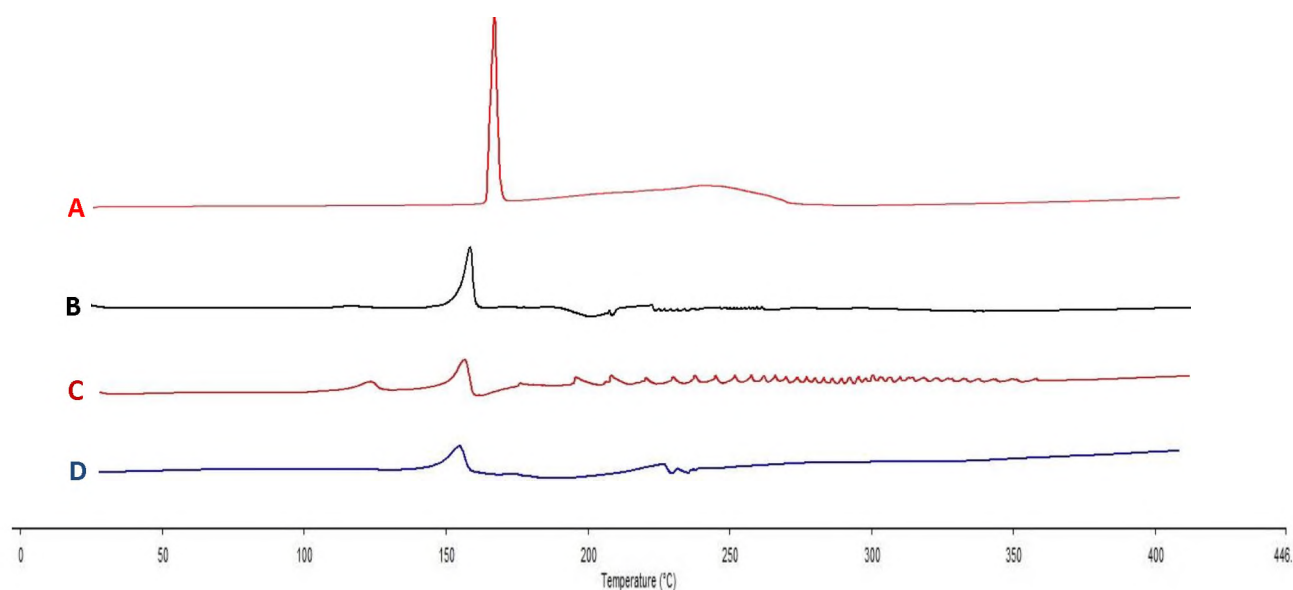


Figure 2.2: DSC thermograms of pure INH (A); physical mixtures of INH + CL (B); INH + cholesterol (C); and of INH + CL + cholesterol (D).

XRD. The XRD patterns of pure INH and its physical mixtures with crude lecithin (CL) and cholesterol (Chol) are compared in **Figure 2.3**. Obviously, all the crystalline peaks of INH appearing at 12 °, 16 °, 17 °, 20 ° and in the range of 25 to 30 ° can be found in the patterns of the physical mixtures. This indicates the crystalline state of INH in the physical mixtures assessed. The outcome from XRD is consistent with DSC regarding the physicochemical compatibility between INH and the lipids.

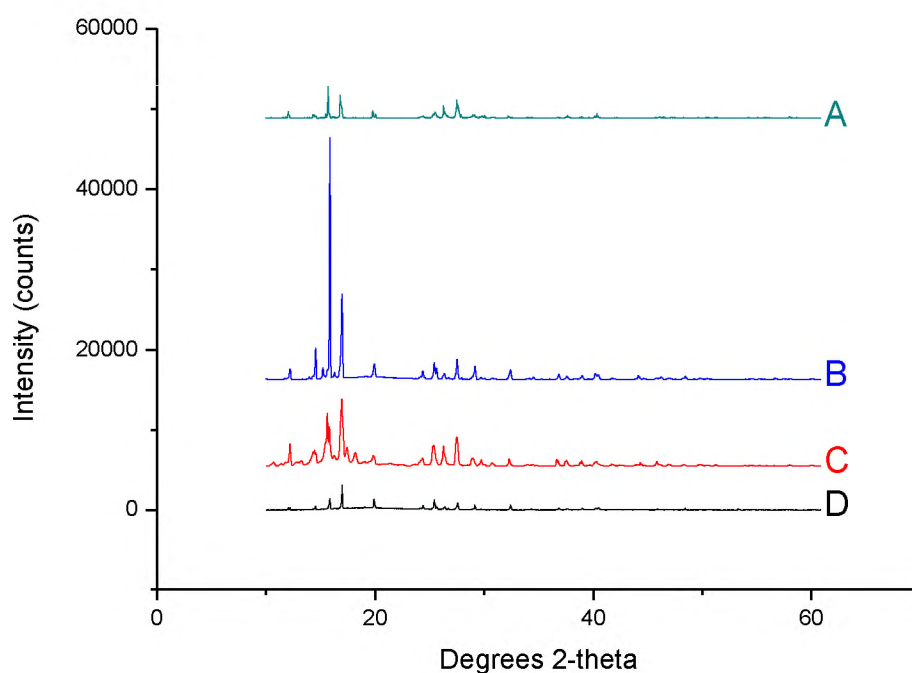


Figure 2.3: Comparison between the XRD patterns of pure INH (A) and the physical mixtures (B: INH + CL; C: INH + Chol and D: INH + CL + Chol).

FTIR. The FTIR spectra of the physical mixtures of INH with crude lecithin (CL) and cholesterol (Chol) are presented in **Figure 2.4** in comparison with the pure drug. **Table 2.2** shows the assignment of the absorption bands to the characteristic stretching vibrations of the functional groups of INH according to Vrabete and Modra (2013). Overall, the FTIR results demonstrate the chemical intactness of INH in all the physical mixtures, as there is no major difference between the wavenumber values found in the spectra compared.

Table 2.2: Absorption bands assigned to the functional groups of INH in the mixtures.

INH functional groups	Wavenumber (cm ⁻¹)			
	Pure INH	INH + Chol	INH + CL	INH+CL+Chol
Pyridin ring	1553	1554	1553	1556
C=C	1602	1603	1603	1603
C=N	1633	1634	1634	1634
C=O	1662	1663	1662	1662
-HN-H	3303	3303	3303	3301

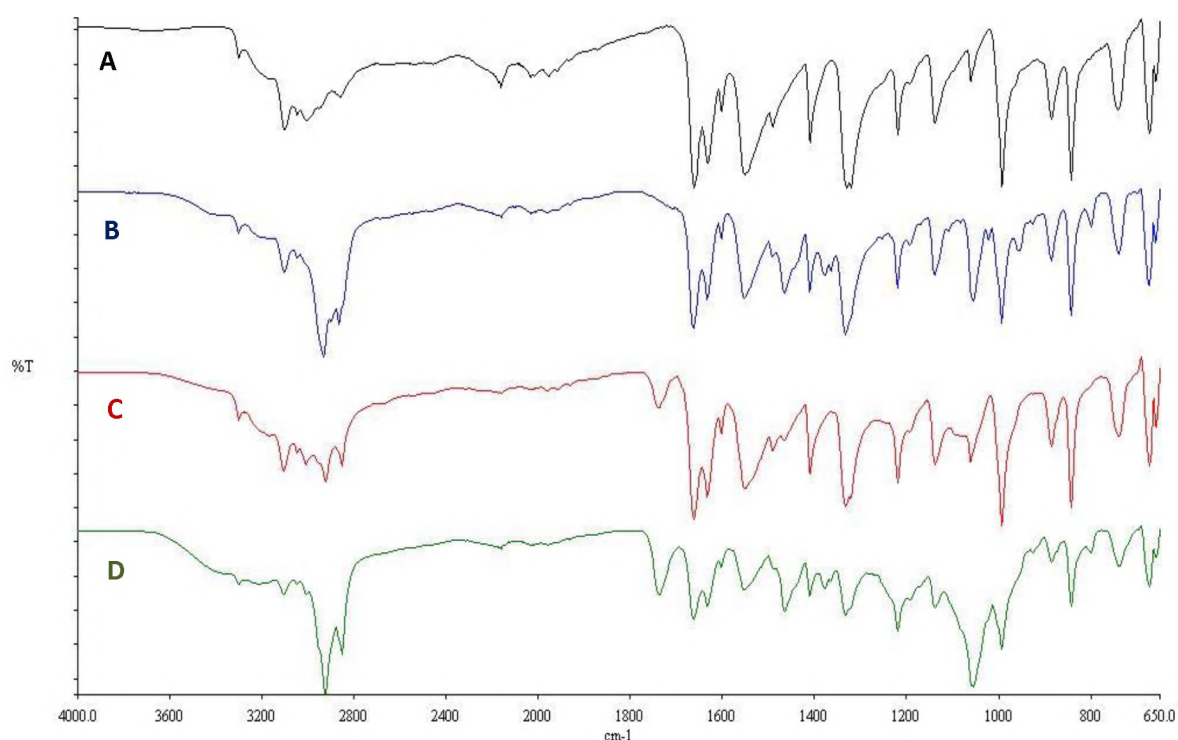


Figure 2.4: FTIR spectra of Pure INH (A); physical mixtures of INH + cholesterol (B); INH + lecithin (C) and INH + cholesterol + lecithin (D).

3.1.2. Evaluation of empty liposomes

3.1.2.1. Particles size and zeta potential

Table 2.3 presents all the first series formulations (S1-F1 to S1-F27) along with their respective average particle sizes and polydispersity index values (PDI). The liposomes with PDI values below 0.6 were evaluated in terms of zeta potential values (ZP). Considering the possible impact of negative ZP on the stability and biological fate of liposomes (Pattni et al., 2015; Nagayama et al., 2007), the formulations that contained highly charged liposomes with relatively acceptable PDI were selected. These included S1-F16 and S1-F18 that showed respective ZP of about -72.0 mV and -70.7 mV; with PDI values of 0.54 and 0.35 respectively. In addition, the average sizes of these two formulations, 607 nm for S1-F16 and 201 nm for S1-F18, were worth improving for the intended application. The graphs of size distribution of these formulations are shown in **Figure 2.5**.

Since lecithin to cholesterol mass ratio (3:1) and lipids to aqueous phase ratio (1:30) were the same for S1-F16 and S1-F18, sonication time was the only factor to be reinvestigated for the second stage of liposome formulation.

Table 2.3: Average particles sizes and PDI values of the first series liposomes along with the some ZP values. *Non-evaluated.

Formulation (S1-F1 to F27)	Lecithin – Chol mass ratio	Lipids – water mass ratio	Sonication time (min)	Average size (nm)	PDI	ZP (mV)
1	1 : 0	1 : 10	15	256.1	0.807	*
2	1 : 0	1 : 10	30	364.3	0.614	*
3	1 : 0	1 : 10	45	294.9	0.859	*
4	1 : 0	1 : 20	15	336.7	0.582	*
5	1 : 0	1 : 20	30	439	0.507	*
6	1 : 0	1 : 20	45	402.6	0.501	*
7	1 : 0	1 : 30	15	563.9	0.559	*
8	1 : 0	1 : 30	30	95.64	0.307	–68.4
9	1 : 0	1 : 30	45	481.9	0.515	–64.0
10	3 : 1	1 : 10	15	395.9	0.797	*
11	3 : 1	1 : 10	30	282.4	0.874	*
12	3 : 1	1 : 10	45	272.6	0.962	*
13	3 : 1	1 : 20	15	644	0.607	*
14	3 : 1	1 : 20	30	283.6	0.511	–75.9
15	3 : 1	1 : 20	45	711.5	0.586	–66.5
16	3 : 1	1 : 30	15	607.2	0.543	–72.0
17	3 : 1	1 : 30	30	467.9	0.559	–50.5
18	3 : 1	1 : 30	45	200.6	0.350	–70.7
19	2 : 1	1 : 10	15	478.5	1.000	*
20	2 : 1	1 : 10	30	389	0.965	*
21	2 : 1	1 : 10	45	575.6	1.000	*
22	2 : 1	1 : 20	15	576.3	0.977	*
23	2 : 1	1 : 20	30	127.2	0.263	–73.8
24	2 : 1	1 : 20	45	235.9	0.401	–61.7
25	2 : 1	1 : 30	15	734.2	0.725	*
26	2 : 1	1 : 30	30	713.7	0.653	*
27	2 : 1	1 : 30	45	421	0.820	*

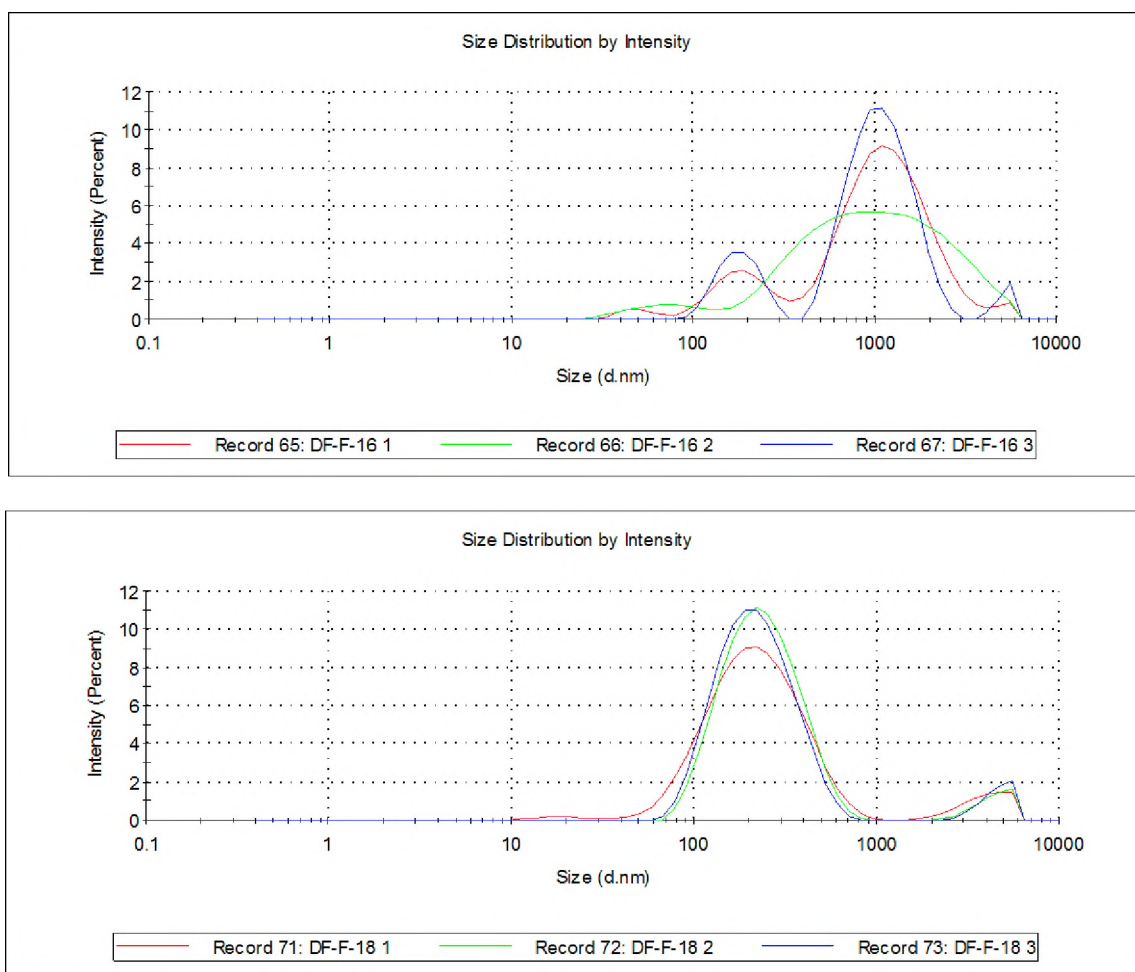


Figure 2.5: Particles size distribution of the liposomal formulations S1–F16 (upper graph) and S1–F18 (lower graph).

The particle sizes and PDI values of the second series liposomes are presented in **Table 2.4**. Among the 27 formulations prepared, the liposomes S2–F12 showed a relatively confined size distribution (PDI 0.27) with an average particle size (501 nm) acceptable for deep lung deposition and macrophage uptake, which both require particle sizes within 500–5000 nm (Iida *et al.*, 2003). The ZP values of three different batches of the formulation S2–F12 evaluated over three consecutive weeks are reported in **Table 2.5**. These liposomes remained highly negatively charged (–51 mV to –61 mV), no substantial variations in ZP could be observed over time. Such negative surface charges (–50 mV to –60 mV) of liposomes have also been reported by Manca *et al.* (2012), who encapsulated rifampicin using soy lecithin and cholesterol in a 3:1 mass ratio. The liposomal formulation S2–F12 was selected for INH encapsulation. The graphs of ZP and sizes distributions of S2–F12 are shown in **Figure 2.6**.

Table 2.4: Average particles sizes and PDI values of the second series liposomes.

Formulation (S2-F1 to 27)	Hydration time (min)	Vortex time (min)	Sonication time (min)	Average size (nm)	PDI
1	30	0	0	677.8	0.539
2	30	0	10	476	0.409
3	30	0	20	415.2	0.501
4	30	5	0	723.5	0.607
5	30	5	10	419.3	0.387
6	30	5	20	209.9	0.318
7	30	10	0	447.5	0.493
8	30	10	10	483	0.388
9	30	10	20	498.1	0.340
10	60	0	0	685.3	0.382
11	60	0	10	494.4	0.296
12	60	0	20	501.8	0.276
13	60	5	0	396	0.306
14	60	5	10	181.4	0.302
15	60	5	20	115.8	0.240
16	60	10	0	423	0.466
17	60	10	10	130.7	0.312
18	60	10	20	181.2	0.324
19	90	0	0	603.8	0.358
20	90	0	10	314	0.460
21	90	0	20	216.1	0.371
22	90	5	0	362.1	0.439
23	90	5	10	503.4	0.355
24	90	5	20	477.5	0.368
25	90	10	0	387	0.401
26	90	10	10	425.2	0.481
27	90	10	20	268.6	0.482

Table 2.5: Average particles size (APS), PDI and zeta potential (ZP) of 3 batches of S2–F12 over 3 weeks.

Batch No	APS ± SD (nm)	PDI ± SD	ZP week 1 (mV)	ZP week 2 (mV)	ZP week 3 (mV)
1	501.8 ± 4.203	0.276 ± 0.023	−56.3 ± 1.260	−52.8 ± 0.416	−59.6 ± 1.270
2	620.7± 15.670	0.318 ± 0.020	−60.0 ± 0.289	−61.6 ± 1.010	−53.6 ± 0.551
3	607.3 ± 9.000	0.280 ± 0.047	−60.2 ± 1.300	−56.3 ± 0.603	−51.9 ± 0.874

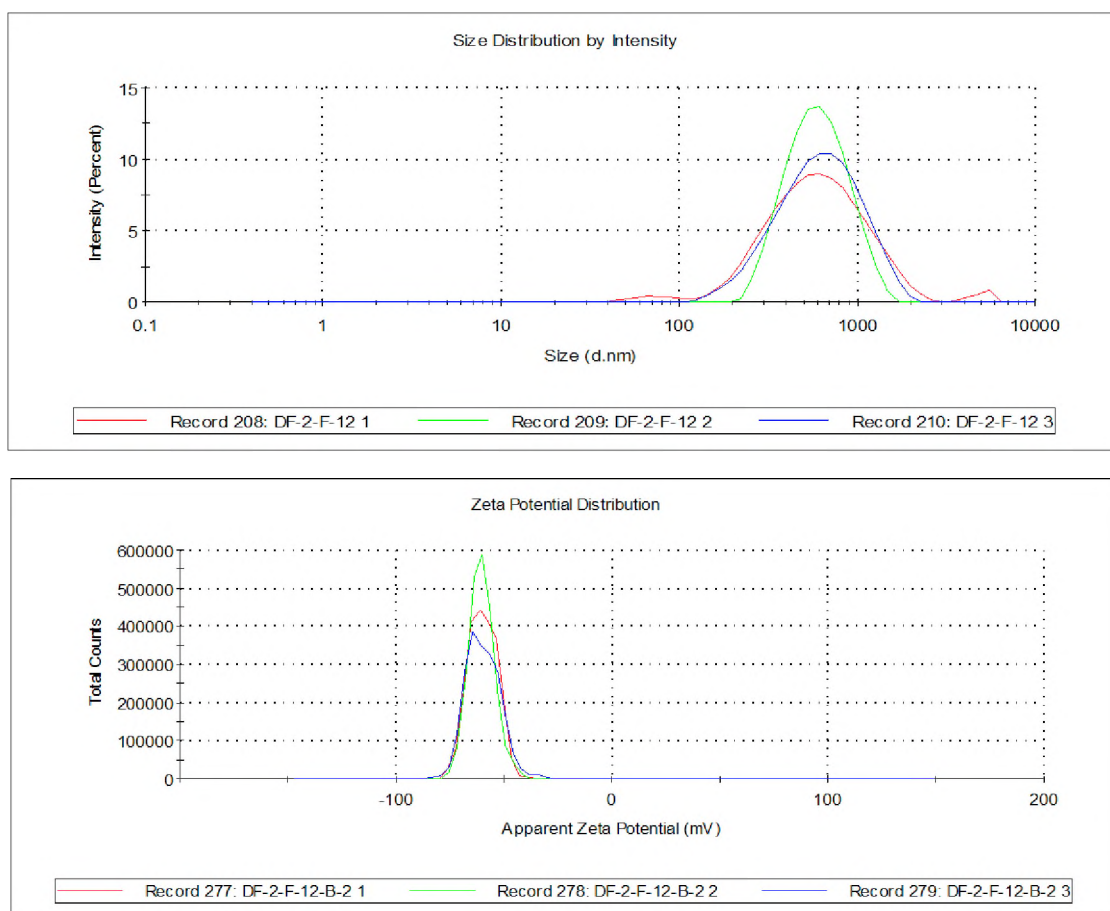


Figure 2.6: Particles size (upper graph) and ZP distributions (lower graph) of the Liposomes S2–F12.

3.1.2.2. Particle shape analysis

Typical TEM micrographs of the liposomes selected in the first preparation series (S1–F16 and S1–F18) and that of the second series S2–F12 are presented in **Figure 2.7**. All the formulations exhibited particles with spherical shape, which is characteristic to liposomes. Additionally, TEM images revealed the presence of liposomes as individual particles, and no aggreates were observed. This might be related to the denseness of surface charges noticed.

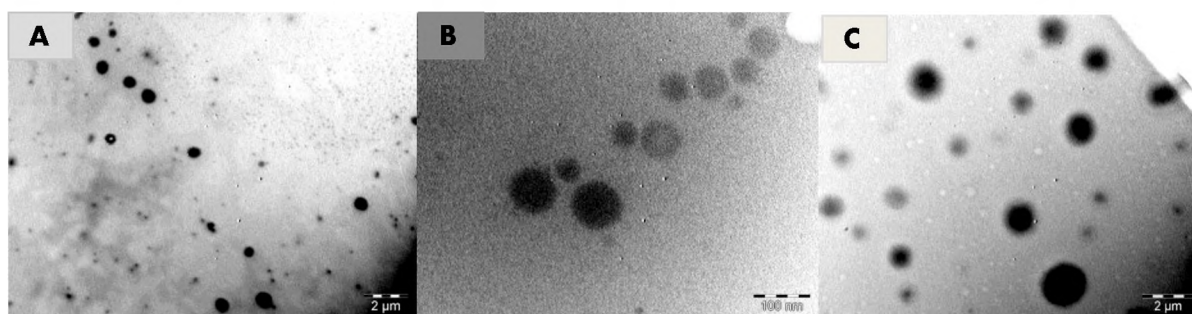


Figure 2.7: Typical TEM micrographs of the liposomes S1–F16 (A) ; S1–F18 (B) and S2–F12 (C).

3.1.3. Validation of the quantification method

Figure 2.8 presents the standard calibration graph obtained by plotting average peak areas against different concentrations of INH. The calibration line established indicates a good linear correlation in the concentration range of 5–500 µg/ml, the obtained correlation coefficient ($R^2 = 0.999$) being in close agreement with the standard value ($R^2 > 0.998$) (Shabir, 2004). The LOD and LOQ were found to be 0.013 µg/ml and 0.042 µg/ml respectively. As presented in **Table 2.6**, the percentage RSD values for intraday analysis show good repeatability, all the values being below 2% (Ayyappa et al., 2011). In addition, the intermediate precision evaluated over six days of experiments was quite acceptable; given that all the RSD values are found bellow 5% (Bartolomeo and Maisano, 2006 and Milán-Segovia et al., 2007). Since the acceptable percentage recoveries range in between 90–110% (Bartolomeo and Maisano, 2006), the accuracy of the present method is quite valid considering the experimental values recorded (**Table 2.6**).

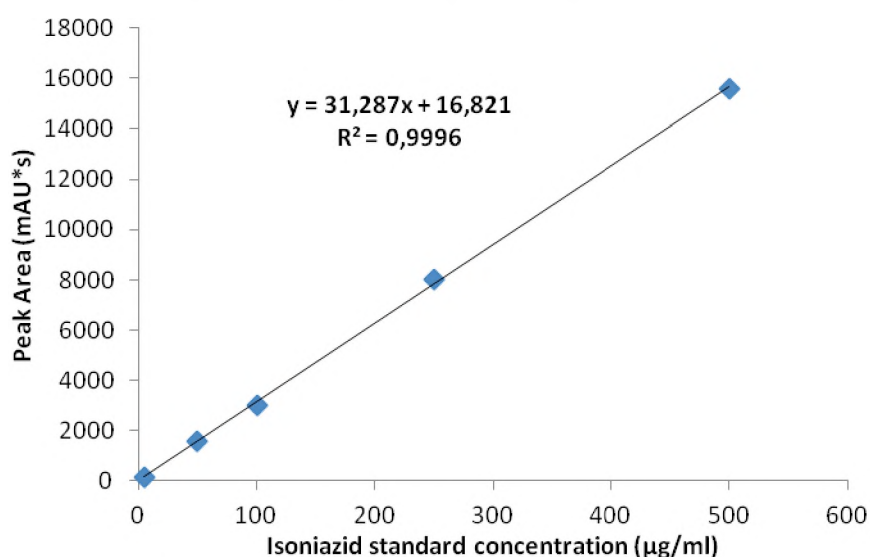


Figure 2.8: Standard calibration plot of isoniazid in the concentration range of 5–500 µg/ml.

Table 2.6: RSD and recovery values over 6 days of experiments.

Concentration (µg/ml)	Intraday precision (%RSD)	Interday precision (% RSD)	Accuracy (% recovery)
5	0.85	3.09	95.39
50	0.33	1.39	101.67
100	0.87	0.53	95.56
250	0.13	0.45	102.42
500	0.15	0.45	99.56

3.2. Characterization of INH-loaded liposomes

3.2.1. Encapsulation efficiency

The means of percentage encapsulation efficiency (%EE) of liposomes made of crude lecithin (CL) are presented in **Table 2.7**. The drug to lipid mass ratio of 2:1 demonstrated the highest value, $78.78 \pm 2.45\%$, after two freeze–thawing cycles. Such a %EE is remarkable as poor encapsulations of hydrophilic drugs in liposomes prepared by film hydration are commonly reported in the literature. Chimote and Banerjee (2010) and Rojanarat et al. (2011) have reported respectively $36.7 \pm 1.8\%$ and 18–30% of EE of INH as a single drug in liposomes. In addition, the entrapment of INH in liposomes using purified lecithin (PL) in the same preparation conditions with CL showed only $20.90 \pm 2.10\%$ of EE. This reinforces the economical status of liposomes made of CL, as the %EE impacts the cost effectiveness of the drug carriers (Kadare et al., 2014). This higher %EE of CL–based liposome might be related to hydrophilic compounds in CL, namely carbohydrates that accommodate INH molecules.

Table 2.7: Percentage EE values for different drug to lipid (CL) mass ratios and freeze–thaw (FT) cycles.

Drug to lipid ratios	EE $\pm$ SD % (n=3)		
	2-FT-Cycles	4-FT-Cycles	6-FT-Cycles
1:1	61.12 $\pm$ 0.18	60.30 $\pm$ 6.10	58.09 $\pm$ 0.68
2:1	78.78$\pm$2.45	77.44 $\pm$ 2.41	55.07 $\pm$ 5.33
4:1	49.54 $\pm$ 7.24	49.44 $\pm$ 2.28	45.77 $\pm$ 1.61

As shown in **Figure 2.9**, the %EE seems to be affected by both the drug to lipid mass ratio and the number of freeze–thawing (FT) cycles. For each ratio, the %EE decreases to some extent with the number of FT cycles. This might be due to a reverse diffusion of INH molecules from liposomes to the dispersing medium throughout prolonged FT, as the membranes remain fluid because of the phase transition of lipids under thawing condition (Costa et al., 2014). In addition, though the increase in drug to lipid ratio was supposed to improve the %EE as a result of increased concentrations gradient, the highest drug to lipid ratio (4:1) showed a mediocre result as compared to other ratios. This might be related to a possible supersaturation of liposomes or/and an increase in the viscosity, which might have affected the diffusion of INH molecules into liposomes during the FT process.

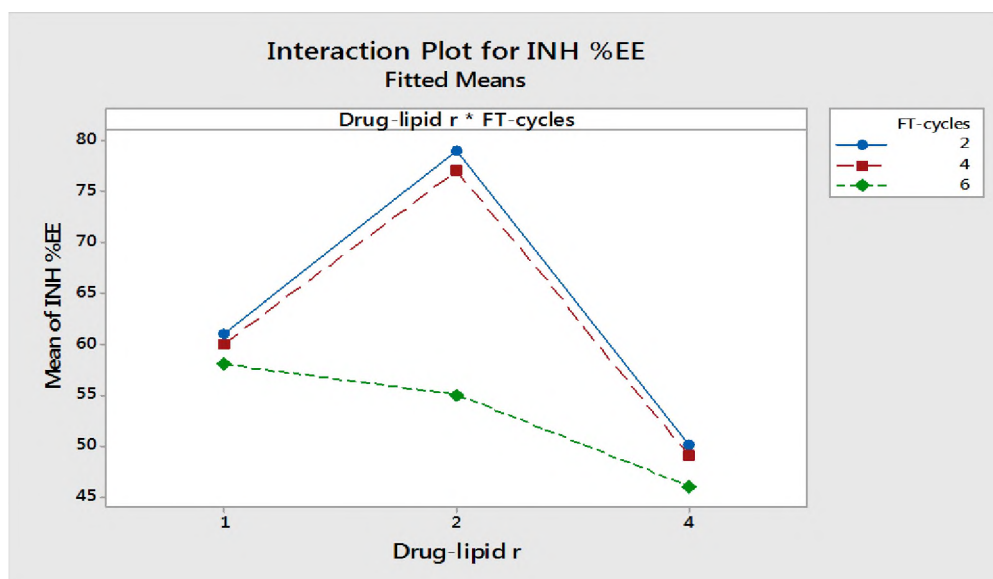


Figure 2.9: Interaction and effect of drug to lipid mass ratio and freeze–thaw (FT) cycles on INH %EE.

3.2.2. Particle size and zeta potential

The results of particle size, poly dispersity index (PDI) and zeta potential (ZP) are presented in **Table 2.8**. Regardless the type of lecithin, all the liposomes showed average particle sizes in the range of 800–1100 nm, which is acceptable for both deep lung deposition and macrophage uptake (Iida et al., 2003). The zeta potential values obtained (from –42.80 mV to –69.10 mV) are valuable for the liposomes stability during the storage (Pattni et al., 2015) and cell internalization, as macrophage scavenger receptors recognize negatively charged particles (Nagayama et al., 2007). The encapsulation of INH seemed to not affect the size and surface charge of liposomes, as no significant differences between empty and INH–loaded liposomes could be observed (p -value > 0.05). Liposomes made of purified lecithin (PL) were more negatively charged than CL–based liposomes. This was expected as the purification process of CL would have increased the proportion of phospholipids (Mertins et al., 2008), which provide negative charges to liposomes (Li et al., 2015). The PDI 0.29 for INH–loaded CL–based liposomes indicates a quite confined particles size distribution.

Table 2.8: Various characteristics of liposomal preparations for triplicate experiments (n=3).

Formulation	Size $\pm$ SD (nm)	PDI $\pm$ SD	ZP $\pm$ SD (mV)	EE $\pm$ SD (%)	Content (%)
INH–CL–Lipo	813.00 $\pm$ 9.21	0.29 $\pm$ 0.06	–42.80 $\pm$ 4.31	78.78 $\pm$ 2.45	61.09 $\pm$ 1.05
Empty CL–Lipo	874.80 $\pm$ 5.15	0.48 $\pm$ 0.07	–47.20 $\pm$ 1.61	–	–
INH–PL–Lipo	1081.00 $\pm$ 20.43	0.43 $\pm$ 0.07	–66.00 $\pm$ 0.65	20.90 $\pm$ 2.10	29.48 $\pm$ 1.87
Empty PL–Lipo	1053.00 $\pm$ 3.02	0.39 $\pm$ 0.10	–69.10 $\pm$ 2.36	–	–

CL: crude lecithin; PL: purified lecithin; Lipo: liposomes; SD: standard deviation.

3.2.3. Particle shape

Throughout TEM observation, the liposomal formulations exhibited spherical particle shape that is characteristic to liposomes. As expected from the zeta potential measured, no particles aggregates were observed; all the liposomes appeared as individual particles. This is consistent with the high surface charges measured, which suggest strong repulsive forces that prevent the aggregation of particles (Pattni et al., 2015). This microscopic analysis value confirms the particle sizes determined by dynamic light scattering (DLS), which is known to give false readings when particles can aggregate. Some typical TEM micrographs of empty and INH-loaded liposomes are presented in **Figure 2.10**.

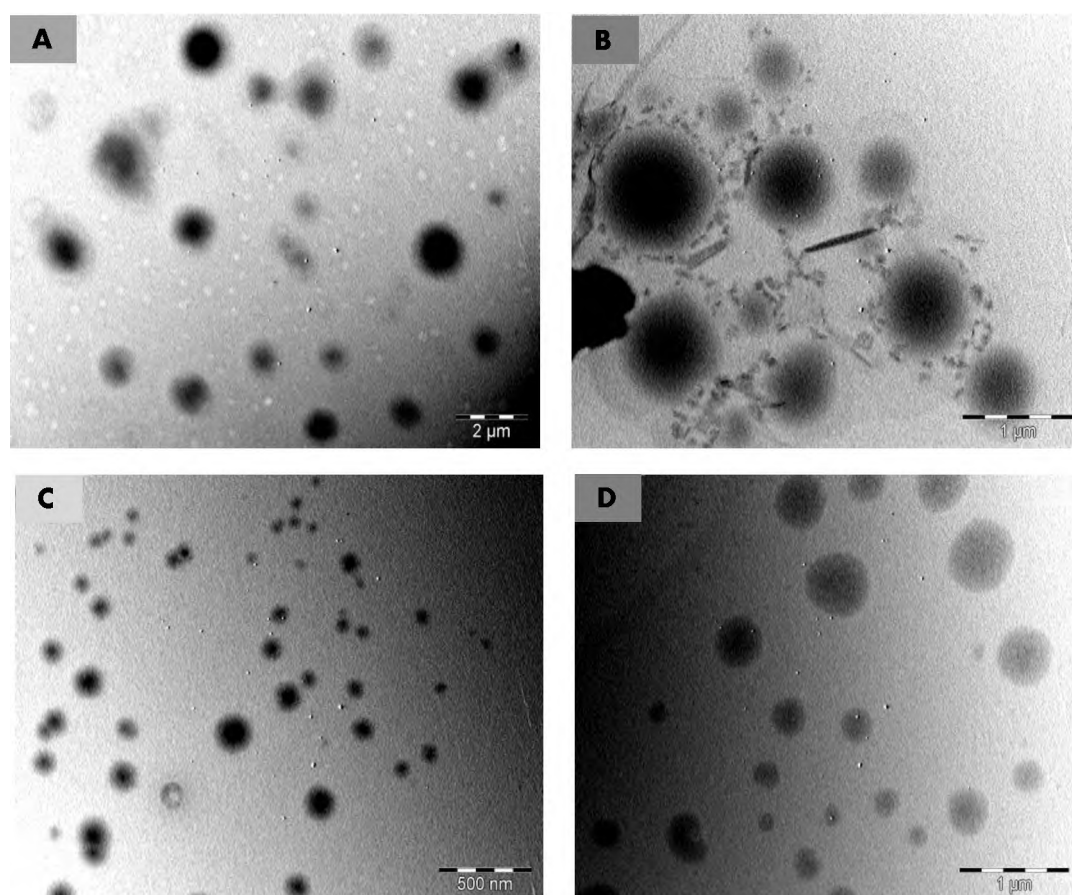


Figure 2.10: TEM micrographs of empty CL-based liposomes (A) ; INH-loaded CL-based liposomes (B); empty PL-based liposomes (C) and INH-loaded PL-based liposomes (D).

3.2.4. Thermal behavior

3.2.4.1. Thermogravimetric analysis

TGA allowed to investigate the thermal stability of INH-loaded CL-based liposomes in comparison with empty CL-based liposomes and pure INH. As shown in **Figure 2.11**, the thermal behavior of INH-loaded CL-based liposomes looks unique, different from that of the empty liposomes and pure INH. The mass loss curve of INH-loaded CL-based liposomes presents mainly two stages of thermal decomposition. The first decomposition occurred from 134.60 to 180.08 °C, where the second one has started and finished at 421.34 °C before the combustion of carbon begins. The only one thermal decomposition stage of pure INH occurred from 171.74 to 305.28 °C; while empty CL-based liposomes decomposed from 244.49 to 410.90 °C. In fact, the earlier thermal decomposition of INH-loaded CL-based liposomes suggests a slight change in the physical state of the materials involved, probably due to the molecular distribution of INH in liposomes.

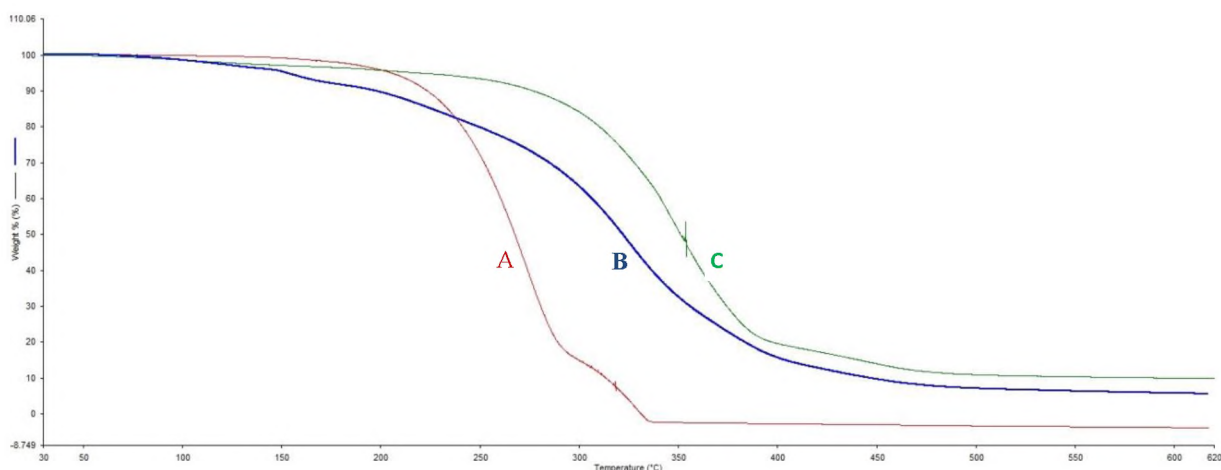


Figure 2.11: TGA mass loss curves of pure INH (**A**), INH-loaded CL-based liposomes (**B**) and Empty CL-based liposomes (**C**).

3.2.4.2. Differential scanning calorimetry

DSC was performed in order to identify the thermal events occurring during the heating process of INH-loaded CL-based liposomes in comparison with empty CL-based liposomes and pure INH. **Figure 2.12** presents all the DSC heat flow curves recorded. The main melting peak around 172.37 °C in DSC curve of pure INH was shifted to about 127.69 °C in INH-loaded CL-based liposomes. This is in close agreement with the early starting decomposition revealed by TGA. This phenomenon is indicative of the loss of some crystalline structure of

INH in liposomes. However, the presence of this characteristic endothermic peak of INH indicates there is no major physicochemical interaction in the formulation, despite the slight change in melting point (Patil et al., 2015).

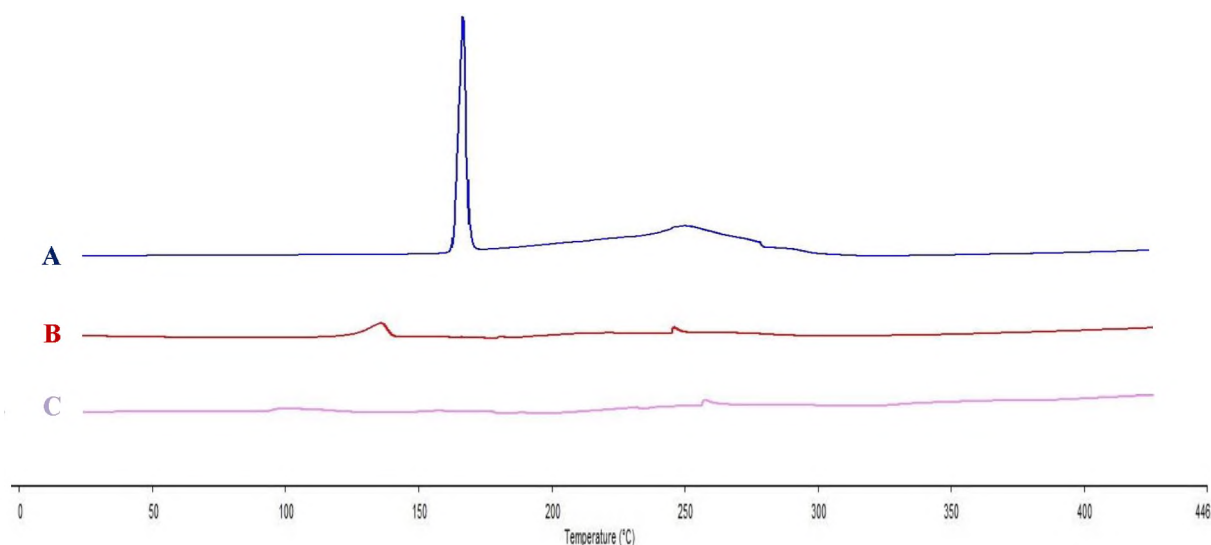


Figure 2.12: DSC Curves of pure INH (A), INH-loaded CL-based liposomes (B) and Empty CL-based liposomes (C).

3.2.5. Evaluation of crystallinity

The XRD patterns of the pure INH, INH-loaded CL-based liposomes and empty liposomes are presented in **Figure 2.13**. While the pattern of empty CL-based liposomes shows a broad amorphous profile, some crystalline structure can be observed in INH-loaded CL-based liposomes. Though the lower diffraction intensity, the characteristic peaks of INH at 12 °, 16 °, 17 °, 20 ° and in the range of 25 to 30° 2-theta appear in the pattern of INH-loaded liposomes. This confirms the presence of INH in liposomes. However, the disappearance of most of the crystalline peaks of INH in the XRD pattern of INH-loaded CL-based liposomes indicates that INH is getting amorphous in liposomes. This strongly supports DSC and TGA results concerning the change in the physical structure of INH in liposomes. Therefore, different physical states of INH in liposomes should be envisaged. INH molecules located in the liposome membranes might be amorphous while those inside of liposomal vesicles might have recrystallized during the freeze-thawing process (Cipolla et al., 2016b). This may explain the broad thermal decomposition of INH-loaded liposomes revealed by TGA. The amorphous structures of INH located in the lipid membranes might have started to decompose before the crystalline ones from internal region of liposomes.

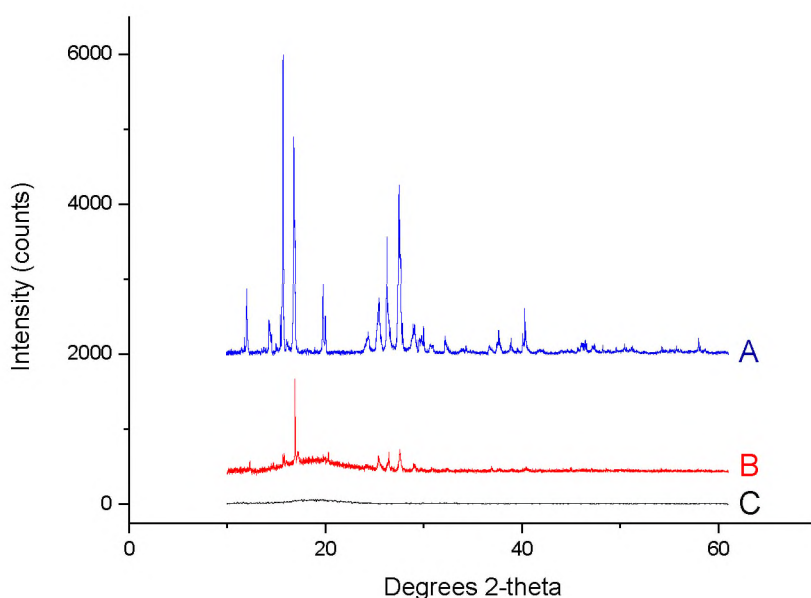


Figure 2.13: XRD patterns of pure INH (A), INH-loaded CL-based liposomes (B) and Empty CL-based liposomes (C).

3.2.6. Fourier transform infrared

FTIR experiments were conducted on the purpose of evaluating the presence and intactness of functional groups of INH in CL-based liposomes. **Figure 2.14** presents the FTIR spectra of the pure INH, INH-loaded CL-based liposomes and empty liposomes. As shown, all the characteristic FTIR absorption bands of INH can be found in the spectrum of INH-loaded CL-based liposomes. The absorption bands from 3107 to 3301 cm^{-1} are assigned to primary and secondary N-H stretching vibrations of hydrazide group of INH. The hydrazide C=O, aromatic N=C and C=C stretches can be found at about 1662 cm^{-1} , 1632 cm^{-1} and 1602 cm^{-1} respectively. The absorption band appearing at 1553 cm^{-1} is characteristic to the pyridine ring of INH (Vrabete and Modra, 2013). This assignment indicates the presence of INH encapsulated in liposomes and can serve as an evidence for chemical stability since all the functional groups are found intact.

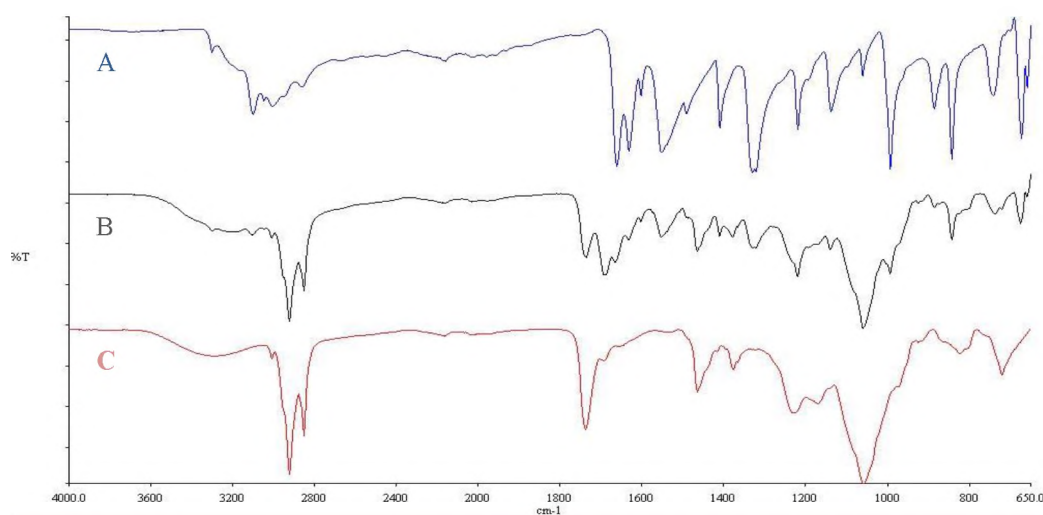


Figure 2.14: FTIR Spectra of pure INH (A), INH-loaded CL-based liposomes (B) and Empty CL-based liposomes (C).

3.2.7. Drug release profile

Data from drug content evaluation and release study conducted *in vitro* in simulated physiological conditions (pH 7.4 phosphate buffers maintained at 37 °C) are presented in **Tables 2.8** and **2.9** respectively. INH-loaded CL-based liposomes have shown higher drug content than liposomes made of purified lecithin (PL-based liposomes), $61.09 \pm 1.05\%$ versus $29.48 \pm 1.84\%$.

Table 2.9: Data from the *in vitro* release of INH.

Time (hour)	%INH-CL-Lipos	%INH-PL-Lipos	%Free INH
0	0	0	0
0,5	35	45	58
1	40	56	70
1,5	41	61	92
2	43	62	93
3	44	63	99
4	46	64	99
5	47	65	100
7	48	66	-
9	49	67	-
12	50	68	-

CL: crude lecithin; PL: purified lecithin; Lipos: liposomes.

A burst drug release was observed after 30 min for all the preparations regardless the lipid type. However, while only 35% of INH was released from CL-based liposomes within 30

min, PL-based liposomes and free INH showed faster release kinetics, with respectively 45% and 58% of INH released after 30 min. As compared to liposomal formulations, the release profile of free INH from the dialysis bag was much quicker, 100% within 5 hours. After 12 hours, PL-based liposomes released up to 68% whereas the percentage release of CL-based liposomes was 50%. Hence, the zero-order release kinetic for CL-based liposomes appears more controlled and sustained than that of PL-based liposomes (**Figure 2.15**).

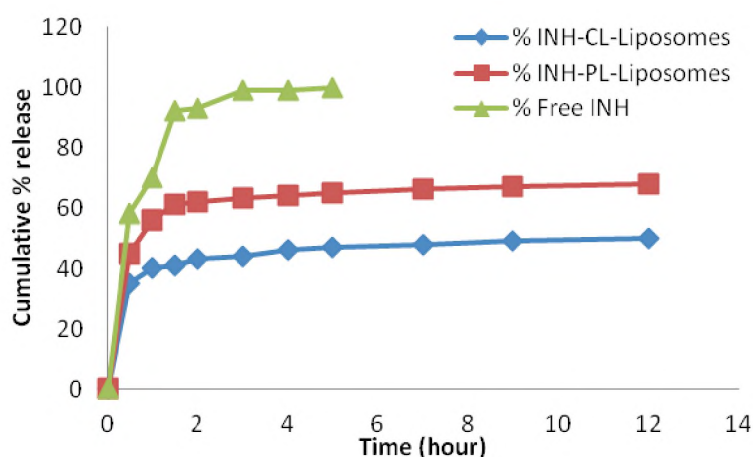


Figure 2.15: Comparison between the zero-order release kinetics of crude lecithin based liposomes (CL-liposomes), purified lecithin based liposomes (PL-liposomes) and free INH.

Overall, CL-based liposomes showed a short rapid release followed by a slower release for the remaining time of the experiment. This release profile estimated can be correlated to the slight change in the physical state of INH in liposomes demonstrated by TGA, DSC and X-RD. Additionally, this kinetic release might be due to the fact that INH is a freely water soluble compound at 25 °C (BP, 2013). Thus, a fast dissolution of INH molecules from liposomal membranes might have occurred at 37 °C, followed by a slow diffusion of INH molecules from the internal core of liposomes. This observation is consistent with the highest R^2 value showed by the Korsmeyer-Peppas model (**Table 2.10**), which indicates that the drug is released from CL-based liposomes by diffusion and erosion mode (Costa and Lobo, 2001). Such a release profile would be beneficially added to several other advantages of CL-based liposomes – such as possible deep lung deposition and macrophage uptake – over the free INH for TB therapy.

Table 2.10: Coefficient of determination (R^2) for the release models of CL-liposomes.

Release model	Zero order	First order	Higuchi	Korsmeyer-Peppas
R^2 value	0.7693	0.8139	0.8909	0.9897

4. CONCLUDING REMARKS

This chapter was focused on the design and development of liposomes suitable for encapsulation of isoniazid (INH) using crude soybean lecithin. The results related to the study of drug–excipients interaction suggested an acceptable physicochemical compatibility between the pure drug and excipients. The liposomal formulation selected showed high encapsulation efficiency of INH ($78.78 \pm 2.45\%$) that has not yet been reported, based on the literature consulted (Bhardwaj et al., 2016; Rojanarat et al., 2011; Chimote and Banerjee, 2010; Gürsoy et al., 2004; Justo and Moraes 2003; Deol et al., 1997). The average particle size (813.00 ± 9.21 nm) and zeta potential (-42.80 ± 4.31 mV) of these INH-loaded liposomes seem to be promising for macrophage–targeting pulmonary delivery (Nagayama et al., 2007; Iida et al., 2003). The release of INH from these liposomes was found to be more controlled and sustained than the profile described by liposomes made of purified lecithin, which besides showed a disappointing encapsulation efficiency ($20.90 \pm 2.10\%$).

The valuable characteristics of INH-loaded liposomes developed suggest the possibility of encapsulating efficiently hydrophilic drugs using crude lecithin as a potential alternative to expensive synthetic or purified natural phospholipids. However, the model drug used in this chapter is currently part of the clinically established multidrug–therapy for active TB disease. As these first–line anti–TB drugs have different solubilities (Sosnik et al., 2010), the following chapter was addressed to the formulation of liposomes containing INH together with a hydrophobic anti–TB drug, rifampicin.

CHAPTER THREE
CO-ENCAPSULATION OF ISONIAZID AND RIFAMPICIN IN LIPOSOMES

A part of this work is under preparation for submission to the Journal of Pharmaceutics and Pharmacology.

1. INTRODUCTION

Rifampicin (RIF) and isoniazid (INH) represent the two mainstays of the therapeutic regimen for latent and active tuberculosis. There is sufficient evidence regarding the potential of RIF to inhibit DNA-dependent RNA polymerase of the intra-macrophage microorganisms, such as *Mycobacterium tuberculosis* (Feklistov et al., 2008; Campbell et al., 2001; Gieffers et al., 1998). The mechanism of action of INH is explained by the inhibition of mycolic acid synthesis and production of active free radicals inside of the mycobacterium (Suarez et al., 2009; Ahmad et al., 2009; Benfang et al., 2000). Although each of these drugs is effective, their combination for TB therapy is highly recommended as monotherapy is often subjected to drug-resistance challenge (WHO, 2015; Yuen et al., 2013; Kumar et al., 2011). RIF coadministered with INH can offer high cure rates in the case of drug-susceptible TB, up to 95–98%. However, this therapeutic success requires a lengthy administration of high doses that exposes the patient to various side effects and leads to inadequate compliance (Manca et al., 2012; Sosnik et al., 2010).

The use of drug delivery systems is an attractive strategy that aims to overcome these pharmacological issues. Liposomes are the most widely used drug carriers for antimicrobial therapy (Zhang et al., 2010). There are some reports discussing the potential of liposomes to encapsulate simultaneously RIF and INH and perform their co-delivery (Gürsoy et al., 2004; Justo and Moraes 2003; Rodrigues et al., 2003; Labana et al., 2002; Deol et al., 1997). Unfortunately, all these liposomal systems are made of expensive synthetic or purified natural lipids that might worsen the costly formulation status of liposomes (Li et al., 2015 and Yokota et al., 2012), which is a limitation for the management of poverty related diseases such as tuberculosis. To the best of our knowledge, there is no report about the use of crude lecithin to co-encapsulate RIF and INH in a single liposomal vehicle.

This chapter aimed to prepare and evaluate a dual liposomal system using crude soybean lecithin for simultaneous delivery of RIF and INH. The following graphical abstract is an illustration of the approach used to co-encapsulate these two drugs (**Figure 3.1**).

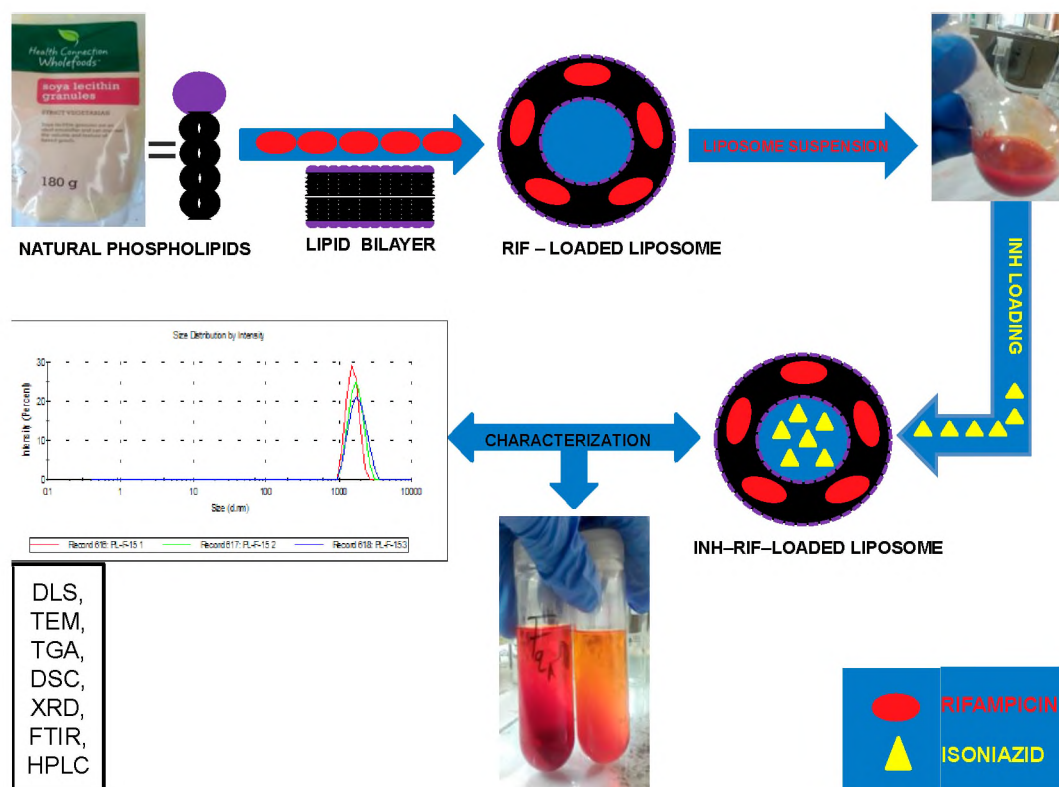


Figure 3.1: Flowchart illustrating the formulation of INH–RIF–loaded liposomes using crude soybean lecithin.

2. EXPERIMENTAL SECTION

2.1. Materials

Isoniazid (INH), rifampicin (RIF), tetraethylammonium bromide (T-Eth.Br), mono- and dibasic sodium phosphates (NaH_2PO_4 and Na_2HPO_4) were purchased from Sigma Aldrich (Germany). Crude soybean lecithin used in this study was from Health Connection Wholefoods (USA). Cholesterol used was from Carlo Erba/Divisione Chimica (Italy). Methanol from Merck (Germany) and acetonitrile from Ranbaxy Fine Chemicals Ltd (India) were of analytical high performance liquid chromatography (HPLC) grade. Ethyl acetate and acetone used were from Protea Chemicals (South Africa). Chloroform was from BM Scientific / Parow Industria (South Africa). All these chemicals were used as purchased without further purifications.

2.2. Validation studies for drugs quantification

The HPLC method for quantification of INH and RIF was validated for linearity, accuracy and precision as described by Mariappan et al. (2000) with a few modifications.

The chromatographic system was consisted of an Agilent HP1100 LC-MSD equipped with a quaternary pump, degasser, DAD detector, 1100 MSD and ChemStation for collection and analysis of data, ZORBAX Elipse Plus C18 4.6 x 150 mm x 5 micro column for reverse phase HPLC analysis. The gradient elution mode was used to achieve the separation. The mobile phase was made of methanol (solvent A) and the aqueous solution of monobasic sodium phosphate 0.01M containing 0.05% w/v T-Eth.Br and the pH was adjusted at 3.5 using orthophosphoric acid (solvent B). Initially, the column was equilibrated for 15 min with 90% of solvent B and 10% of solvent A. Two minutes after the first injection, the composition of the solvent B was proportionally decreased by 3% per minute during 15 minutes. Then, the proportion of solvent A was increased at the rate of 1% per minute for the remaining time of the experiment, 40 minutes. Before the next injection, the initial composition was restored for 15 minutes, and the gradient elution proceeded as described. All the solutions were filtered using 0.45 μ m Millipore filters and 20 μ l was injected in triplicate, and the means of peak areas were considered for quantification. The flow rate was set at 1 ml/min and the detection system was UV light at 245 nm.

Linearity. A stock solution containing both INH and RIF at the concentration of 1 mg/ml was prepared in methanol. From this solution, appropriate dilutions were carried out to make solutions of INH and RIF at the concentrations of 5; 10; 50; 100 and 500 μ g/ml. The linearity of the HPLC method was then evaluated by analyzing the diluted solutions prepared.

Accuracy. Two separate solutions of INH and RIF at 1 mg/ml were used to make combined diluted solutions of INH 150 μ g/ml and RIF 225 μ g/ml in methanol. The experiment was conducted in triplicate and a freshly established calibration curve was used to calculate respective concentrations. The recovery was evaluated based on the actual concentrations, considering the stock solution analyzed beforehand.

Precision. A combined solution of RIF and INH 1 mg/ml was prepared every day to make two diluted solutions of 150 and 225 μ g/ml. These solutions were analyzed in triplicate for the assessment of repeatability, intraday precision. The intermediate precision was evaluated on 3 days experiments using freshly prepared solutions, interday precision. The percentage relative standard deviation (%RSD) was calculated to assess the precision following standard values.

2.3. Preparation of dual liposomes

RIF and INH were encapsulated simultaneously in liposomes. The Minitab software (version 12) was used for full factorial design of experiments for 9 preparations made of three different mass ratios of crude soybean lecithin (CL) to cholesterol (Chol) and lipids to RIF (**Table 3.1**).

Table 3.1: Different mass ratios of crude lecithin (CL) to cholesterol (Chol) and lipids to RIF.

Levels	CL-Chol mass ratio	lipids-RIF mass ratio
1	1 : 0	1 : 0.25
2	1 : 0.33	1 : 0.5
3	1 : 1	1 : 1

Based on the encapsulation efficiency, three CL-based liposomes were selected and their various equivalent liposomes were prepared using the purified soybean lecithin (PL) previously prepared for comparison purpose. The compositions of all the liposomal formulations prepared in this chapter are presented in **Table 3.2**. As a hydrophobic drug, RIF was treated along with the lipid components; while INH was incorporated in pre-prepared liposomes from an aqueous solution by freeze-thaw loading technique.

Table 3.2: Description of the composition of liposomal preparations.

Formulation	Lecithin type	Lecithin-Chol mass ratio	Lipid-RIF mass ratio	Lipid-INH mass ratio
CL-F1	Crude	1 : 0	1 : 0.25	1 : 2
CL-F2	Crude	1 : 0	1 : 0.5	1 : 2
CL-F3	Crude	1 : 0	1 : 1	1 : 2
CL-F4	Crude	1 : 0.33	1 : 0.25	1 : 2
CL-F5	Crude	1 : 0.33	1 : 0.5	1 : 2
CL-F6	Crude	1 : 0.33	1 : 1	1 : 2
CL-F7	Crude	1 : 1	1 : 0.25	1 : 2
CL-F8	Crude	1 : 1	1 : 0.5	1 : 2
CL-F9	Crude	1 : 1	1 : 1	1 : 2
CL-F10	Crude	1 : 0	1 : 0.25	1 : 0
CL-F11	Crude	1 : 0	1 : 0	1 : 2
CL-F12	Crude	1 : 0	1 : 0	1 : 0
PL-F13	Purified	1 : 0	1 : 0.25	1 : 2
PL-F14	Purified	1 : 0.33	1 : 0.25	1 : 2
PL-F15	Purified	1 : 1	1 : 0.25	1 : 2
PL-F16	Purified	1 : 0	1 : 0.25	1 : 0
PL-F17	Purified	1 : 0	1 : 0	1 : 2
PL-F18	Purified	1 : 0	1 : 0	1 : 0

2.3.1. Encapsulation of rifampicin

Appropriate amounts of the lipid components (total of 200 mg) and RIF were dissolved in chloroform (2 ml) in a clean 25 ml round bottom flask. The organic solvent was removed at 60 °C for 5 min using a rotary evaporator (Büchi Rotavapor R-205, Switzerland). The flask was removed and stored in a vacuum desiccator at room temperature overnight. Millipore water (6 ml) was added for hydration of the lipid film at 60 °C under stirring at 400 rpm for 60 min. The red suspension obtained was transferred to a 50 ml centrifuge tube and Millipore water (3 ml) was used to rinse the flask and give a volume level for centrifugation. The preparation was subjected to low speed centrifugation (Beckman Coulter Allegra 64R) at 4,000 rpm for 5 min in order to allow free RIF particles to settle (Patel et al., 2009). The supernatant containing RIF-loaded liposomes was decanted off and kept for further steps. The experiment was done in triplicate and any liposomal formulations free from RIF were prepared in similar conditions except the addition of this drug.

2.3.2. Incorporation of isoniazid

The liposomal supernatant obtained above was transferred to a Beckman centrifuge tube containing INH (400 mg). The mixture was gently shaken and warmed in order to speed up drug dissolution. Thereafter, the centrifuge tube was placed into a bath sonicator (Digital Ultrasonic Cleaner, Spellbound 909) set at 60 °C for 20 min. The preparation was then subjected to two cycles of freeze thawing by soaking the tube in the liquid nitrogen for 5 min and in oil bath set at 60 °C for 10 min (Costa et al., 2014). The frozen-thawed liposomes were allowed to equilibrate at room temperature for 60 min and Millipore water (20 ml) was added to produce a volume level for centrifugation. The centrifugation of this mixture was done at 20,000 rpm for 20 min using Beckman Coulter Allegra 64R at room temperature. The supernatant was decanted off and kept for the evaluation of INH encapsulation efficiency. The residual liposomes isolated were rinsed five times with Millipore water (20 ml) and freeze-dried (Lyo Lab 3000, Apollo Scientific cc) to produce a solid liposomal preparation that was kept away from light at room temperature for further evaluations. Triplicate experiment was performed and any INH-free liposomes were prepared as described except the addition of this drug.

2.4. Characterization of dual liposomes

2.4.1. Determination of encapsulation efficiency

2.4.1.1. Encapsulation efficiency for rifampicin

The free RIF isolated by centrifugation was treated with HPLC grade methanol (30 ml) and diluted to 1/10 for quantification using the validated HPLC method (section 2.2). To calculate the encapsulation efficiency (EE), a preparation free from lipid components was made following the same liposomes preparation procedure to produce a RIF–control solution. The quantification of this solution provided a theoretical total concentration of RIF, considering the concentration of the above free RIF as un–encapsulated drug concentration. The percentage EE (%EE) was evaluated in triplicate following the equation bellow, as described by Costa et al. (2014) and Singh et al. (2011):

$$\%EE = \frac{\text{Theoretical concentration} - \text{free drug concentration}}{\text{Theoretical concentration}} \times 100 \quad (3.1)$$

2.4.1.2. Encapsulation efficiency for isoniazid

The supernatant isolated during INH incorporation was diluted to 1/10 using HPLC grade methanol. A control solution of INH was prepared and treated in the same conditions, starting by dissolving INH (400 mg) in the above lipid–free RIF–control solution. This was done in order to estimate the theoretical total concentration of INH to be used along with the concentration of free INH in the current supernatant for calculating the encapsulation efficiency (EE). The quantification was done in triplicate using the HPLC method validated for simultaneous analysis of INH and RIF. Any trace of RIF detected in this supernatant was evaluated and considered as part of the un–encapsulated RIF. The percentage EE (%EE) of INH in liposomes was calculated according to the equation (3.1) presented above.

2.4.2. Determination of particle size and zeta potential

All the freeze–dried preparations were re–dispersed in Millipore water for particle size and zeta potential analysis. The measurements were taken in triplicate using either an ordinary cuvette or a capillary cuvette depending on the determination, particle size or zeta potential respectively. Zetasizer nano ZEN–3600 MAL1043132 (Malvern Instruments) was used for the measurement at room temperature under the scattering angle of 173°.

2.4.3. Particle shape analysis

The shape and dispersion of liposomal particles were evaluated by microscopic observation using transmission electron microscopy instrument (Zeiss Libra–120KV TEM instrument). The liposomal suspension from particle size and zeta potential analysis was placed on the copper grid drop wise. The liquid in excess was absorbed with a piece of filter paper. The samples were allowed to dry for 48 hours before the TEM experiment.

2.4.4. Differential scanning calorimetry

DSC was used for the study of the polymorphism of the liposomal preparations. The samples were scanned in the temperature range of 30 to 445 °C at a flow rate of 10 °C/min. The atmosphere of the sample chamber was saturated with nitrogen gas flowing at the rate of 20 ml/min. PerkinElmer DSC-6000 instrument that gives upward endothermic peaks was used and data were processed using the software DSC Pyris series.

2.4.5. X-Ray Diffraction

The crystalline state of the two drugs co-encapsulated was assessed by XRay. The free drugs, mono-drug-loaded liposomes and empty liposomes were studied along for comparison purpose. The XRD patterns were recorded on Bruker D8 Discover instrument equipped with a Lynx Eye detector, using a nickel filter and Cu-K α radiation set at 1.5404 angstrom. The samples were placed on a glass sample holder and the scans were run in the 2-theta range of 10 to 100 °, with a slit width of 6.0 mm and a step size of 1 °/min.

2.4.6. Fourier transform infrared spectroscopy

The intactness of the functional groups of the encapsulated drugs was evaluated by FTIR. The FTIR spectra of all the freeze-dried samples were measured on a PerkinElmer Spectrum 100 FTIR Spectrometer and the data were processed using the software FTIR Spectrum. For each measurement, 8 scans were run in the frequency range of 4000 to 650 cm⁻¹.

2.4.7. Drug release study

The liposomal formulations that showed higher %EE (CL-F1, CL-F4 and CL-F7) were selected for drug release study along with the mono-drug loaded liposomes (CL-F10 and CL-F11) and free drugs for comparison purposes. The drug content evaluation and release study were conducted in triplicate following the modified procedures described by Rojanarat et al. (2011) and Chimote & Banerjee (2010) for pulmonary delivery.

Fifty mg of the samples was weighed in a 10 ml volumetric flask and phosphate buffer (pH 7.4) was added to the mark. The obtained preparation was allowed to incubate for 45 min at room temperature and gently shaken to produce a homogenous liposomal dispersion. On one hand, 1 ml of this suspension was placed in a 10 ml volumetric flask and the volume was filled to the mark using methanol. The mixture was sonicated (Digital Ultrasonic Cleaner, Spellbound 909) for 30 min at 35 °C to promote liposomes destruction and drug dissolution. The clear solution obtained was filtered 5 times through 0.45 µm syringe filters and the validated HPLC method (section III.2.2) was used for the quantification of the two drugs using freshly established calibration curves. The content of each drug (%DC) was calculated using the following equation:

$$\%DC = \frac{\text{Amount of drug recovered}}{\text{Amount of the formulation used}} \times 100 \quad (3.2)$$

On the other hand, 1 ml of the above liposomal dispersion was loaded into a well tighten dialysis bag (Membra-Cel MD 10-14 x 100 CLR, Sigma Aldrich) that was placed in a size-10 glass vial containing 20 ml of the buffer (pH 7.4) and a magnetic stirrer. The release medium was maintained at 37 °C under stirring at 240 rpm (Cooke et al., 2005). At specific time intervals (0.5, 1, 1.5, 2, 3, 4, 5, 7, 9 and 12 hours), 5 ml of the draining medium was withdrawn for drug quantification using the validated HPLC method described in section 2.2. After every sampling, an equivalent volume of the fresh buffer was added in order to maintain sink condition. The release profile of the free drugs from the dialysis bag was also studied in the same conditions and all the experiments were replicated. The major release models, namely zero order, first order, Higuchi and Korsmeyer-Peppas models; were evaluated in order to characterize the release mechanism of the liposomes CL-F1. The respective coefficients of determination (R^2) were calculated, and the model with the highest R^2 value was considered for allocation of the release mechanism (Costa and Lobo, 2001).

3. RESULTS AND DISCUSSION

3.1. Validation of the method for quantification

Linearity. The standard calibration plots of both INH and RIF demonstrate a linear correlation between the concentrations, in the range of 5–500 µg/ml, and the chromatographic analytical signal, peak areas (**Figure 3.2**). The correlation coefficients (R^2) recorded, $R^2 = 0.9993$ for INH and $R^2 = 0.9991$ for RIF, are in close agreement with the literature value, $R^2 > 0.998$ (Shabir, 2004).

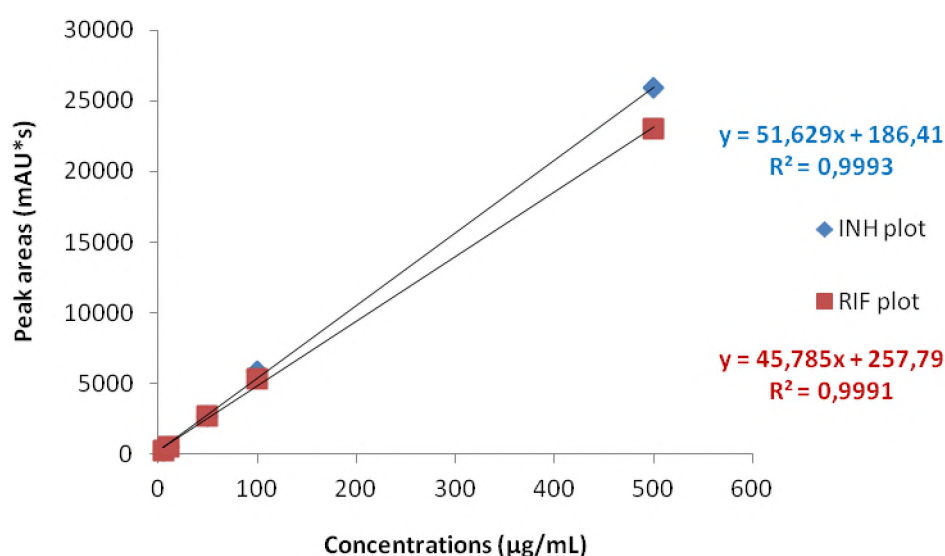


Figure 3.2: Standard calibration plots of INH and RIF.

Accuracy. The results for the assessment of accuracy presented in **Table 3.3** show that the HPLC method used is accurate, as all the percentage recovery values are within the acceptable range defined in the literature, 90–110% (Bartolomeo and Maisano, 2006). The typical chromatogram presented in **Figure 3.3** shows a net separation of INH and RIF by the gradient elution system set on the C18 HPLC column used. The retention time for INH was 1.6 ± 0.2 min while RIF was eluted after 29.9 ± 1.1 min.

Table 3.3: Data from accuracy assessment for standard INH and RIF.

Drug	Actual Concentration (µg/ml)	Calculated Concentrations (µg/ml)	Accuracy (%Recovery)
INH	150	149.9	99.9
	225	228.8	101.7
RIF	150	151.4	100.9
	225	230.9	102.6

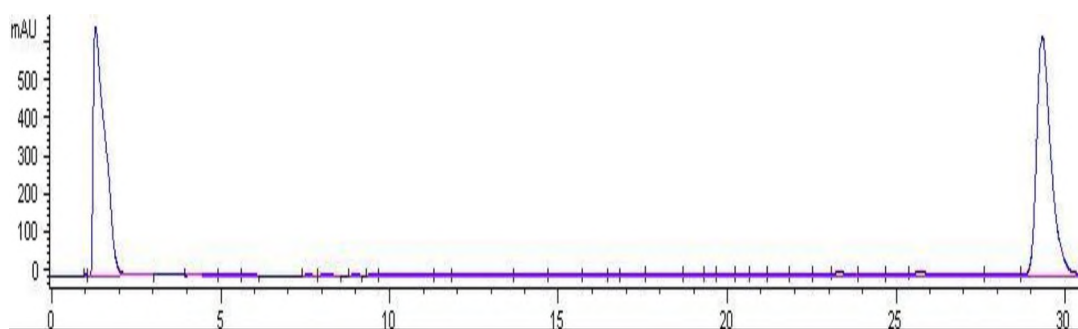


Figure 3.3: Typical HPLC chromatogram obtained using a gradient elution system on a C18 column for the separation and quantification of INH and RIF.

Precision. The results of the experiments conducted to evaluate the precision of the HPLC method are presented in **Table 3.4**. As all the values of the relative standard deviation (RSD) were in accordance with the literature values, the method was deemed to be acceptable in terms of both repeatability, $RSD < 2\%$ (Ayyappa et al., 2011), and intermediate precision, $RSD < 5\%$ (Milán-Segovia et al., 2007; Bartolomeo and Maisano, 2006).

Table 3.4: RSD values for repeatability (n=3) and intermediate precision on 3 days experiments.

Drugs	Concentration ($\mu\text{g/ml}$)	Intraday Precision (%RSD)	Interday Precision (%RSD)
INH	150	1.18	1.14
	225	0.98	1.77
RIF	150	1.79	1.67
	225	1.24	1.20

3.2. Encapsulation efficiency

The percentage encapsulation efficiency (%EE) of all the batches of liposomes are presented in **Table 3.5**. For the first nine preparations, the %EE values for the two drugs seem to be affected by lecithin to cholesterol mass ratio and RIF to lipids mass ratio. The %EE of RIF tends to decrease with the increase of the amount of cholesterol (**Figure 3.4**). Patil et al. (2015) also reported the same observation while encapsulating RIF alone in liposomes using soy lecithin. As for INH, **Figure 3.5** shows that the %EE of INH seems to decrease when the mass of RIF increases. These phenomena can also be observed for the liposomes made of purified lecithin, PL-F13 to PL-F15. A probable explanation might be the fact that liposomal membranes can get saturated when accommodating RIF and cholesterol simultaneously. This

possible saturation can also affect the loading of INH in liposomes by freeze thawing, as the process involves the diffusion of drug molecules into liposomes (Costa et al., 2014).

Table 3.5: Average particle sizes (PS), polydispersity index (PDI), zeta potential (ZP) and encapsulation efficiency (EE) of liposomes with the respective standard deviations (SD), n=3.

Formulation	PS $\pm$ SD (nm)	PDI $\pm$ SD	ZP $\pm$ SD (mV)	EE $\pm$ SD (%)	
				INH	RIF
CL-F1	1114.00 $\pm$ 42.37	0.34 $\pm$ 0.06	-63.30 $\pm$ 2.13	59.03 $\pm$ 0.68	90.10 $\pm$ 1.15
CL-F2	905.40 $\pm$ 80.25	0.50 $\pm$ 0.13	-69.80 $\pm$ 1.05	28.53 $\pm$ 0.43	32.90 $\pm$ 0.19
CL-F3	1095.00 $\pm$ 69.97	0.61 $\pm$ 0.14	-74.10 $\pm$ 4.27	26.61 $\pm$ 0.52	18.59 $\pm$ 0.86
CL-F4	894.10 $\pm$ 18.30	0.44 $\pm$ 0.03	-50.80 $\pm$ 1.48	57.13 $\pm$ 2.03	64.93 $\pm$ 4.51
CL-F5	964.10 $\pm$ 30.58	0.51 $\pm$ 0.07	-57.10 $\pm$ 2.89	33.69 $\pm$ 1.87	27.11 $\pm$ 0.78
CL-F6	947.10 $\pm$ 40.88	0.64 $\pm$ 0.05	-62.00 $\pm$ 1.23	30.53 $\pm$ 0.50	17.93 $\pm$ 0.60
CL-F7	1514.00 $\pm$ 132.40	0.36 $\pm$ 0.11	-48.04 $\pm$ 0.93	45.65 $\pm$ 1.52	55.24 $\pm$ 2.80
CL-F8	1365.00 $\pm$ 45.32	0.37 $\pm$ 0.06	-56.10 $\pm$ 0.50	36.23 $\pm$ 3.28	22.80 $\pm$ 0.87
CL-F9	1236.00 $\pm$ 37.42	0.42 $\pm$ 0.05	-64.90 $\pm$ 0.73	32.91 $\pm$ 4.87	12.45 $\pm$ 0.44
CL-F10	594.10 $\pm$ 5.88	0.35 $\pm$ 0.09	-59.60 $\pm$ 2.10	—	66.33 $\pm$ 0.15
CL-F11	568.00 $\pm$ 8.89	0.35 $\pm$ 0.18	-20.30 $\pm$ 0.71	53.21 $\pm$ 1.33	—
CL-F12	546.80 $\pm$ 0.46	0.54 $\pm$ 0.04	-57.90 $\pm$ 2.68	—	—
PL-F13	758.60 $\pm$ 3.61	0.43 $\pm$ 0.04	-45.50 $\pm$ 3.39	43.91 $\pm$ 0.59	78.69 $\pm$ 1.63
PL-F14	799.10 $\pm$ 29.67	0.44 $\pm$ 0.07	-47.60 $\pm$ 1.40	30.44 $\pm$ 3.15	48.59 $\pm$ 1.72
PL-F15	1530.00 $\pm$ 112.80	0.19 $\pm$ 0.02	-47.80 $\pm$ 3.14	27.06 $\pm$ 2.90	41.62 $\pm$ 3.00
PL-F16	543.50 $\pm$ 14.13	0.61 $\pm$ 0.02	-56.00 $\pm$ 1.08	—	59.99 $\pm$ 0.13
PL-F17	687.80 $\pm$ 87.07	0.71 $\pm$ 0.19	-35.60 $\pm$ 4.80	34.65 $\pm$ 2.43	—
PL-F18	513.30 $\pm$ 22.08	0.61 $\pm$ 0.03	-48.20 $\pm$ 0.88	—	—

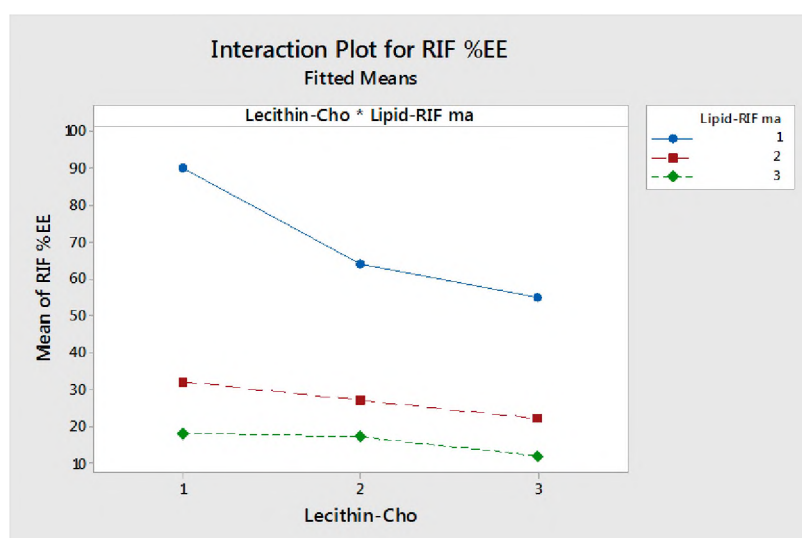


Figure 3.4: Effects of crude lecithin to cholesterol (Cho) and lipids to RIF mass ratios on the %EE of RIF.

Among all the liposomal formulations, CL-F1 exhibited the highest %EE for both INH and RIF; $59.03 \pm 0.68\%$ and $90.10 \pm 1.15\%$, respectively. This result sounds attractive considering some similar works reported in the literature. Deol et al. (1997) have co-encapsulated INH and RIF in liposomes made of purified eggs lecithin and distearoylphosphatidylethanolamine chemically modified with polyethylene glycol. These stealth liposomes showed 8–10% and 44–49% of EE for INH and RIF, respectively. When developing a dual liposomal system using dipalmitoylphosphatidylcholine (DPPC), Gürsoy et al. (2004) have achieved %EE of $73.84 \pm 0.78\%$ and $81.53 \pm 2.06\%$ for INH and RIF respectively. Interestingly, the lipid to drug mass ratios used by this group were 1:0.027 for INH and 1:0.000027 for RIF, whereas in CL-F1 INH and RIF were co-encapsulated in the lipid to drug mass ratios of 1: 2 and 1:0.25 using crude soybean lecithin. With one part of the lipid material, larger amounts of drugs have been encapsulated in CL-F1, as compared to DPPC-based liposomes. Considering the impact of the entrapment capacity on the cost of the drug carriers (Kadare et al., 2014), this feature of CL-F1 would add more economical advantage to the use of crude soybean lecithin in liposomal drug delivery.

On the other hand, liposomes made of purified lecithin (PL-F13 to PL-F17) showed lower %EE of the two drugs as compared to their equivalent formulations based on crude lecithin; CL-F1, CL-F4, CL-7, CL-F10 and CL-F11. Regardless the lipid type, the dual liposomal systems showed higher %EE than mono-drug loaded liposomes (p -value <0.05). This is consistent with the results reported by Gürsoy et al. (2004) and suggests the existence of some advantageous molecular interactions between the two drugs that might promote their simultaneous loading in liposomes.

The mass ratio of INH to RIF used in the liposomal formulation CL-F1 was 8:1. The possibility of loading higher amount of INH in liposomes may be related to its hydrophilic nature and small size. These features might have allowed INH molecules to be accommodated in both aqueous and hydrophobic regions while RIF is mainly dispersed into lipid membranes (Rodrigues et al., 2003). The %EE of INH in CL-F1 ($59.03 \pm 0.68\%$) was not as high as the one previously obtained when INH was encapsulated alone in the presence of cholesterol ($78.78 \pm 2.45\%$). Despite this observation, the present result seems to be still substantial considering the %EE of INH in liposomes reported by Rojanarat et al. (2011) and Chimote & Banerjee (2010), 18–30% and $36.7 \pm 1.8\%$ respectively. As for RIF, the %EE showed by CL-F1 ($90.10 \pm 1.15\%$) is significantly higher (p -value <0.05) than the results reported by Patil et al. (2015) and Manca et al. (2012), respectively 79.25% and $76 \pm 4\%$.

3.3. Particle size and zeta potential

The average values of particles size, polydispersity index (PDI) and zeta potential for all the liposomal formulations are presented in **Table 3.5**. Overall, the mean particles sizes for the liposomes prepared were found between 513.30 ± 22.08 and 1530.00 ± 112.80 nm. This is attractive as the particles with sizes in the range of $0.5\text{--}5\text{ }\mu\text{m}$ can be subjected to both deep lung deposition and uptake by alveolar macrophages (Iida *et al.*, 2003). Interestingly, the liposomal formulations CL-F1, CL-F3, CL-F7, CL-F8, CL-F9 and PL-F15 showed the average particles sizes within the range $1\text{--}3\text{ }\mu\text{m}$ reported to be optimum for these two biological phenomena (El-Sherbiny *et al.*, 2015). The mean particles sizes for the first nine liposomal preparations were found to be randomly and slightly different (**Figure 3.5**).

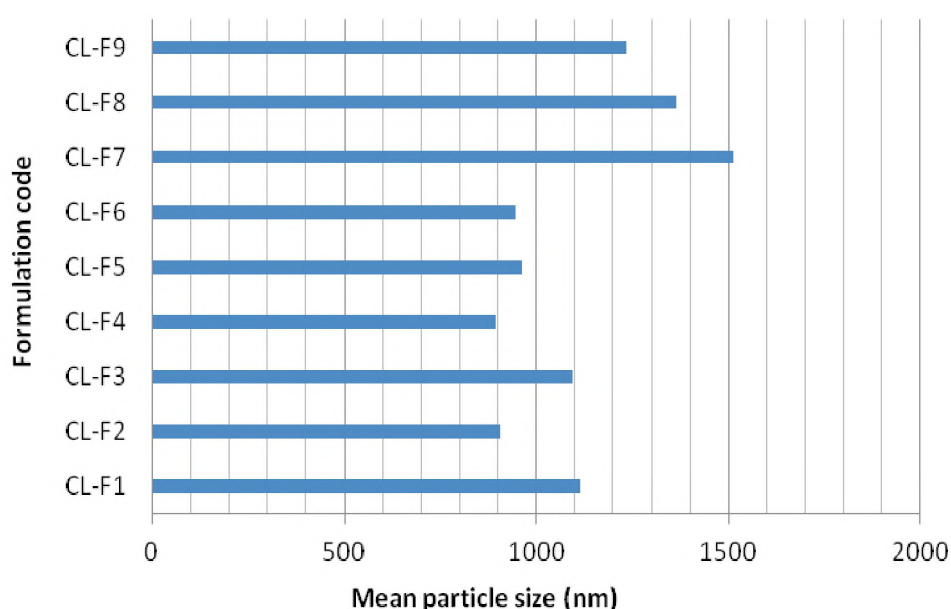


Figure 3.5: Comparison between mean sizes of the first nine liposomes made of crude soybean lecithin.

Regardless the type of lecithin, the dual liposomes seem to be significantly bigger than mono-drug loaded liposomes and empty liposomes as shown in **Figure 3.6** ($p\text{-value} < 0.05$). The PDI values of the liposomes prepared were found between 0.19 ± 0.02 and 0.71 ± 0.19 . Typical size distribution by intensity graphs (**Figure 3.7**) show relatively confined size distributions of liposomes CL-F1, CL-F10, CL-F11 and CL-F12.

The mean values of zeta potential measured were found in between -20.30 ± 0.71 mV and -74.10 ± 4.27 mV (**Table 3.5**). While no significant differences in zeta potentials can be observed between empty and drugs-loaded liposomes, liposomal formulations containing

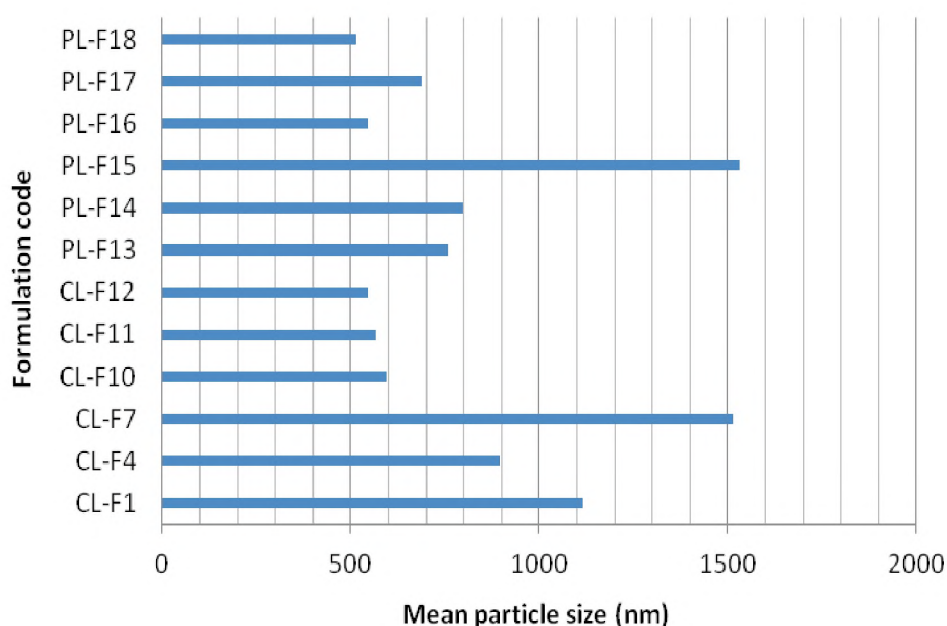


Figure 3.6: Comparison between mean sizes of liposomes made of crude lecithin with equivalent liposomes made of purified soybean lecithin.

INH alone seem to be poorly charged, -20.30 ± 0.71 mV for CL-F11 and -35.60 ± 4.80 mV for PL-F17. This might be caused by the presence of INH molecules in the polar regions of the vesicle membranes (Rodrigues et al., 2003), which might have created an interference with the surface charge of liposomes. Interestingly, the dual liposomal formulation that exhibited the highest %EE for the two drugs (CL-F1) showed a highly negative zeta potential value, -63.30 ± 2.13 mV. Indeed, this zeta potential observed is a promising feature for both shelf life and half-life of liposomes. Surely, highly charged particles tend to be dispersed individually in the preparation as a result of repulsive forces implied, which therefore promote the stability during the storage (Pattni et al., 2015). As for the biological fate, the zeta potential recorded is promising for the internalization of these liposomes by macrophages, since scavenger receptors of macrophages recognize negatively charged particles (Nagayama et al., 2007).

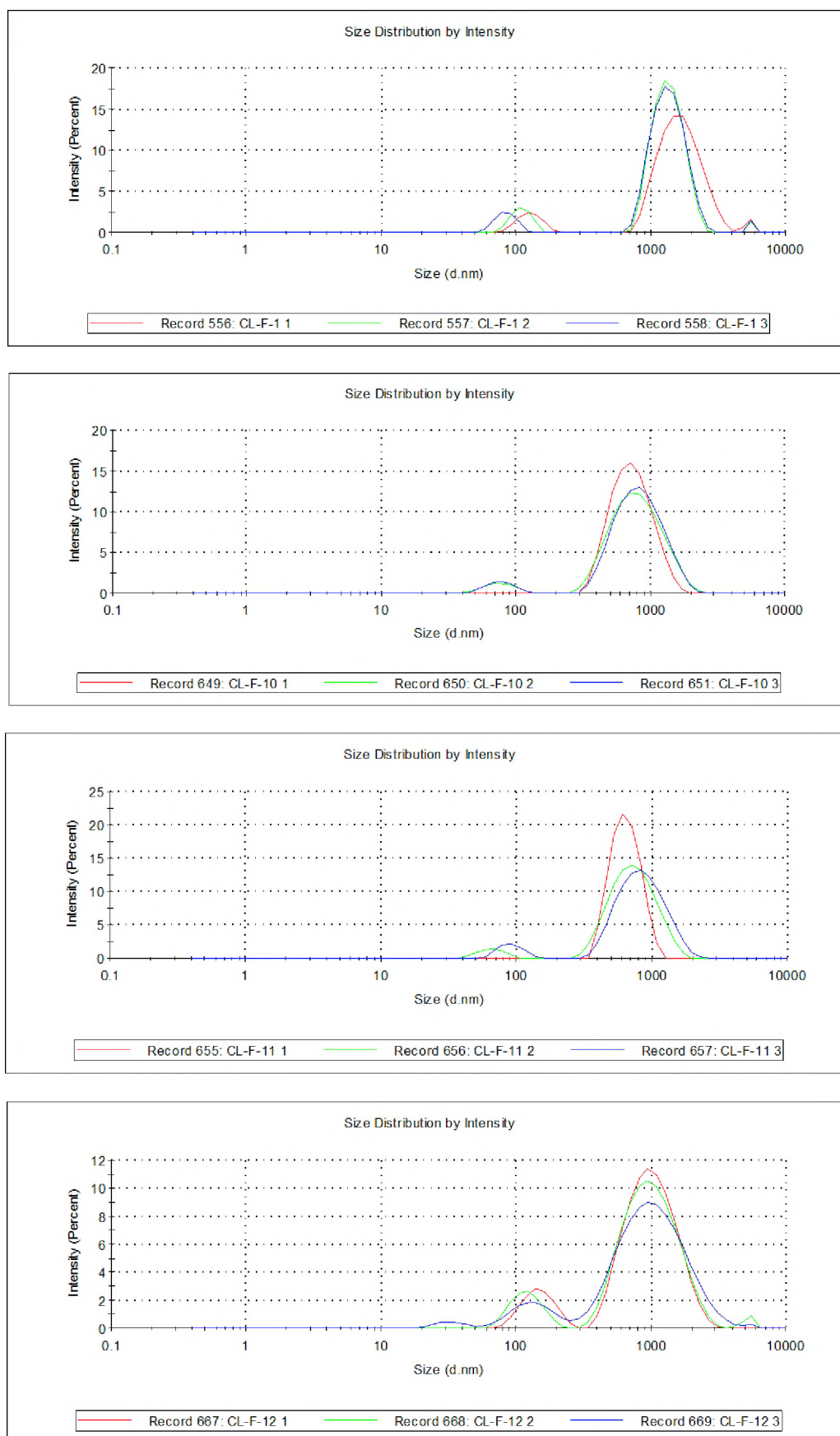


Figure 3.7: Typical graphs of size distribution for liposomes CL-F1, CL-F10, CL-F11 and CL-F12.

3.4. Particle shape

During TEM visualization, all the preparations showed individual particles with spherical shape, which is indicative of the presence of liposomal vesicles. The absence of particles aggregates during the microscopic analysis suggests the existence of strong repulsive forces between liposomes, probably due to the denseness of negative charges (zeta potential) revealed. This provides some credit to the results of particle size determined by dynamic light scattering (DLS), which is often limited by the phenomenon of particles aggregation (Pattni et al., 2015). **Figure 3.8** presents typical TEM micrographs of some liposomes made of crude soybean lecithin (CL-F1, CL-F10, CL-F11 and CL-F12).

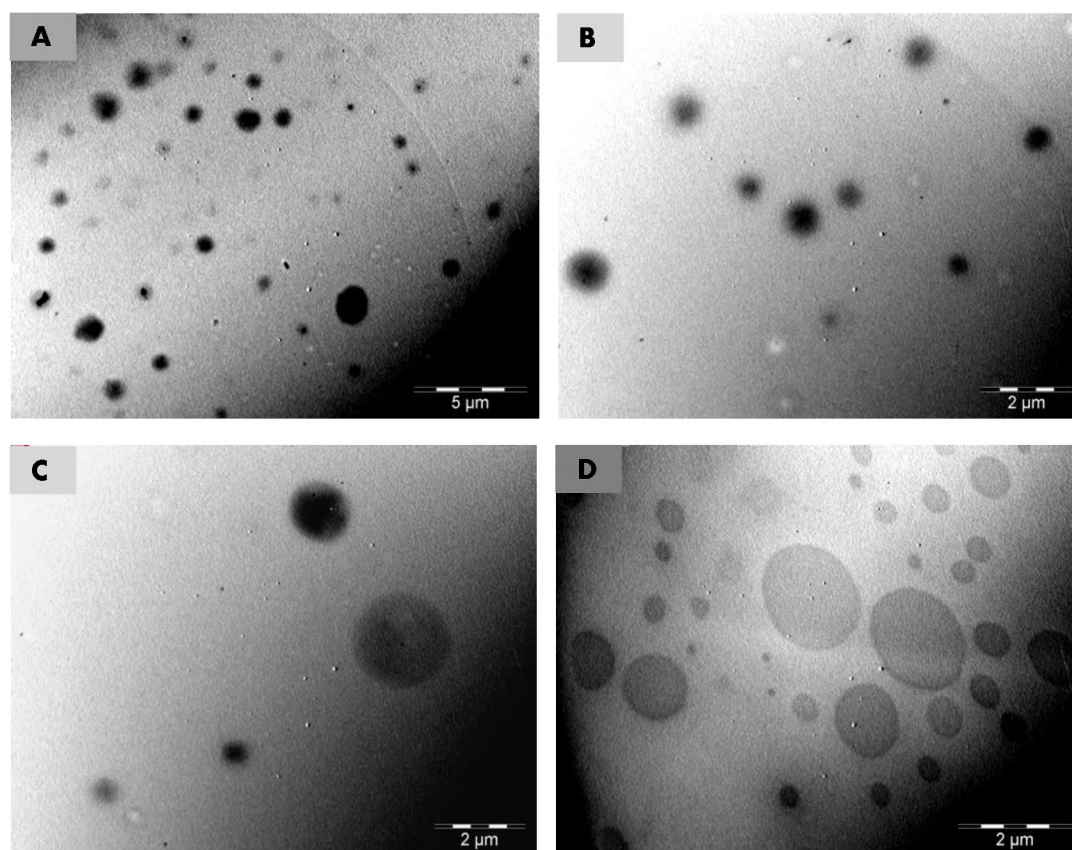


Figure 3.8: Typical TEM micrographs of crude lecithin based liposomes CL-F1 (A), CL-F10 (B), CL-F11 (C) and CL-F12 (D).

3.5. Thermal stability

The DSC curve of the liposomes CL–F1 shows two major characteristic peaks corresponding to the melting of the two drugs encapsulated (**Figure 3.9**). The first melting point at 121.29 °C can be allocated to RIF and the second at 165.71 °C to INH based on the peak areas measured, 17.20 J/g and 63.03 J/g respectively, which can be correlated to the content of each drug.

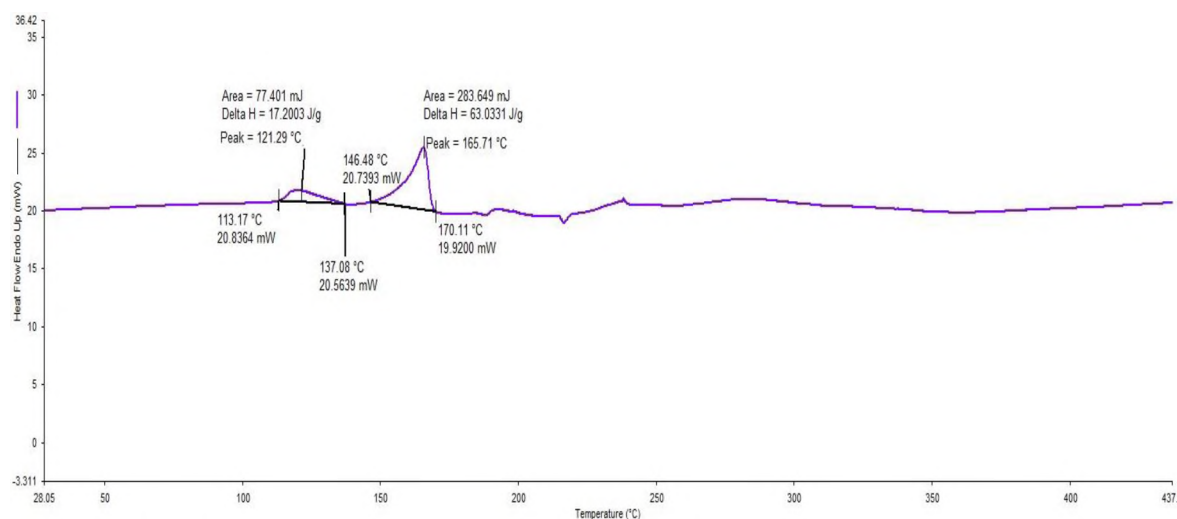


Figure 3.9: DSC thermogram of the liposomal formulation CL–F1.

The major data from DSC experiments are presented in **Table 3.6**. The melting points observed in the heat flow curves of the pure drugs (**Figures 3.10A** and **3.10G**) were shifted to lower values in the DSC thermogram of CL–F1 (**Figure 3.10C**), the difference in melting temperatures being 72.00 °C for RIF and 9.66 °C for INH. The decrease in melting points suggests that the two drugs are getting amorphous in liposomes (Singh et al., 2015; Patil et al., 2015). The empty liposomes (CL–F12) showed a melting peak around 287.92 °C as shown in **Figure 3.10D**. As for the DSC thermogram of the physical mixture of crude lecithin and the two drugs (1:1:1), the melting peak of RIF did not show up while INH showed almost the same behavior (**Figure 3.10E**). Since the melting peak of lipids was still present at around 248.93°C, the disappearance of the peak of RIF might be only related to its molecular distribution in lecithin but not to any major interactions (Patil et al., 2015). While INH showed only a slight change in melting behavior in mono–drug loaded liposomes CL–F11 (**Figure 3.10B**), the melting peak of RIF in the DSC curve of CL–F10 (**Figure 3.10F**) was still significantly different to that of the pure drug (p–value <0.05). This might be related to

the fact that RIF's molecules have been deeply embedded in liposomal membranes as suggested by Rodrigues et al. (2003).

Table 3.6: DSC data showing the melting (MT), onset and end temperatures of the drugs along with enthalpy.

Sample	INH				RIF			
	Onset (°C)	MT (°C)	End (°C)	Enthalpy (J/g)	Onset (°C)	MT (°C)	End (°C)	Enthalpy (J/g)
CL-F1	146.48	165.71	170.11	63.03	113.17	121.29	137.08	17.20
CL-F10	—	—	—	—	117.16	123.10	137.30	16.23
CL-F11	162.00	170.21	177.59	73.24	—	—	—	—
Pure INH	169.83	174.37	181.32	207.23	—	—	—	—
Pure RIF	—	—	—	—	179.52	193.29	200.69	30.17
Drugs+Lecithin	149.82	167.06	173.75	49.42	—	—	—	—

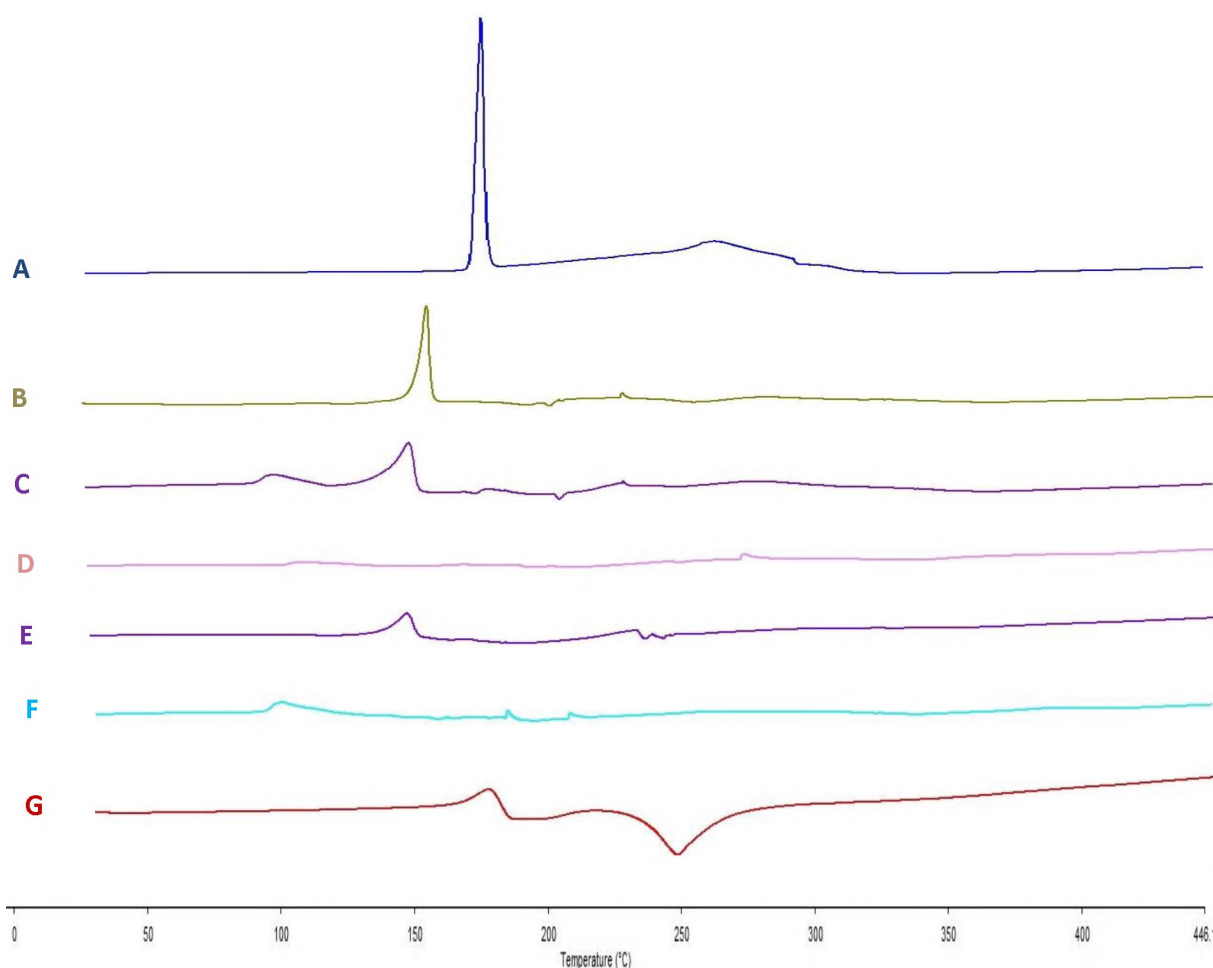


Figure 3.10: DSC thermograms of pure INH (A); CL-F11 (B); CL-F1 (C); CL-F12 (D); 1:1:1 physical mixture of RIF+INH+ crude lecithin (E); CL-F10 (F) and pure RIF (G).

3.6. Evaluation of crystallinity

The XRD patterns of the formulations CL-F1, CL-F10, CL-F11, CL-F12, pure INH and RIF are presented in **Figure 3.11**. The liposomes CL-F1 showed major crystalline peaks at positions 15–18 °, 20 ° and 25–30 °. These might be related to the presence of INH that has also exhibited almost the same diffraction profile as pure drug, given that the pure RIF showed some characteristic sharp peaks at 17–20 ° and 20–25 ° 2-theta that are not seen in the pattern of CL-F1. This can be also clearly observed when the comparison involves the mono-drug loaded liposomes. While the XRD profile of liposomes CL-F11 is almost similar to that of the dual liposomes, CL-F10 has shown the same halo pattern with the empty liposomal formulation CL-F12. This supports and confirms the loss of crystallinity of RIF in liposomes revealed by DSC. The present observation is also consistent with the results reported elsewhere in the literature regarding the change in the physical state of RIF in liposomes (Patil et al., 2015; Singh et al., 2015). Such an amorphous state would be useful to promote drug release since, as a hydrophobic drug, the dissolution of RIF in aqueous media is already limited (Sosnik et al., 2010).

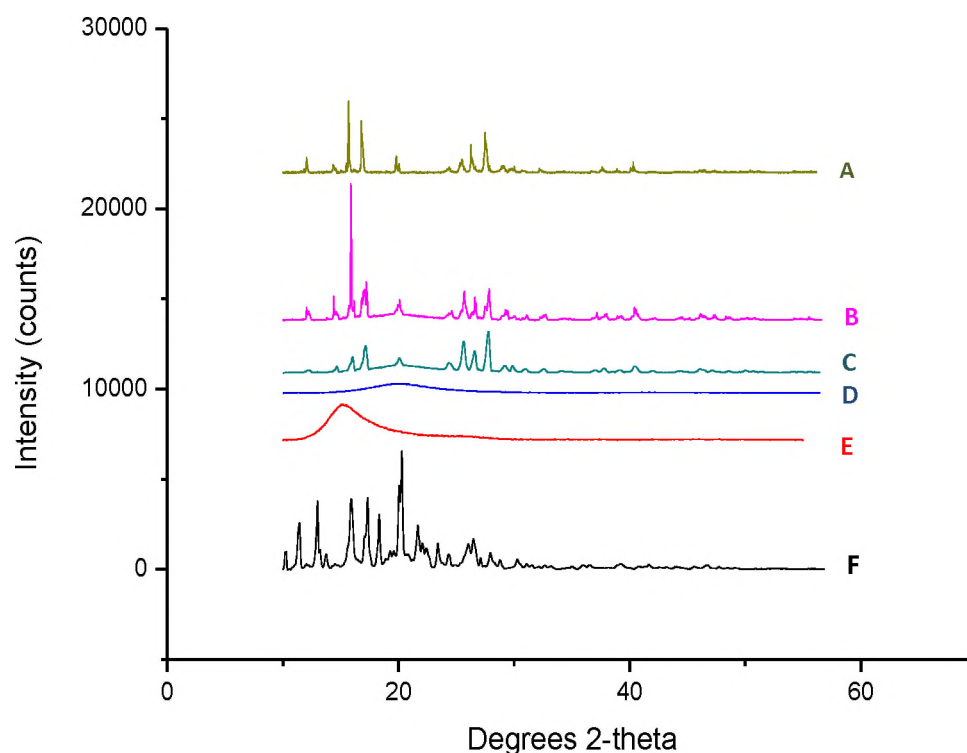


Figure 3.11: Comparison between the XRD patterns of pure INH (A); CL-F11 (B); CL-F1 (C); CL-F12 (D); CL-F10 (E) and pure RIF (F).

3.7. Fourier transform infrared

Figure 3.12 presents a detailed FTIR spectrum of the liposomal formulation CL-F1. The absorption bands labeled with the respective frequency values correspond to various functional groups of the compounds found in this liposomal formulation. **Figure 3.13** compares the FTIR spectra of the pure drugs to those of the formulation CL-F1 and the corresponding physical mixture in a 1:1:1 mass ratio. The characteristic peaks of RIF appeared at 3418 cm^{-1} , 1734 cm^{-1} , 1653 cm^{-1} , 1563 cm^{-1} , 1428 cm^{-1} and 1249 cm^{-1} ; which correspond to the respective stretching vibrations of alcohol O–H, acetyl C=O, asymmetric C=N, C=C, C–N and ether C–O–C (Singh et al., 2015). Nevertheless, these functional groups might not be specific to RIF in CL-F1, as most of them can be found either in lipids or carbohydrates that compose crude soybean lecithin or in INH. This can be observed in the spectrum of the physical mixture where the absorption bands seem to be concealed or/and subjected to the additive effects. However, the absorption band that appears at 1552 cm^{-1} is characteristic to the pyridine ring of INH (Vrabete and Modra, 2013), which can be also characterized by the N–H stretching vibrations of hydrazide appearing in the range of $3108\text{--}3303\text{ cm}^{-1}$. All these absorption bands can be observed in the spectra of CL-F1 and physical mixture. Since no strange bonds can be detected in the studied spectra, it appears worth suggesting that no major drug–drug or drug–lipids molecular interactions occurred and the encapsulated drugs are chemically intact (Patil et al., 2015).

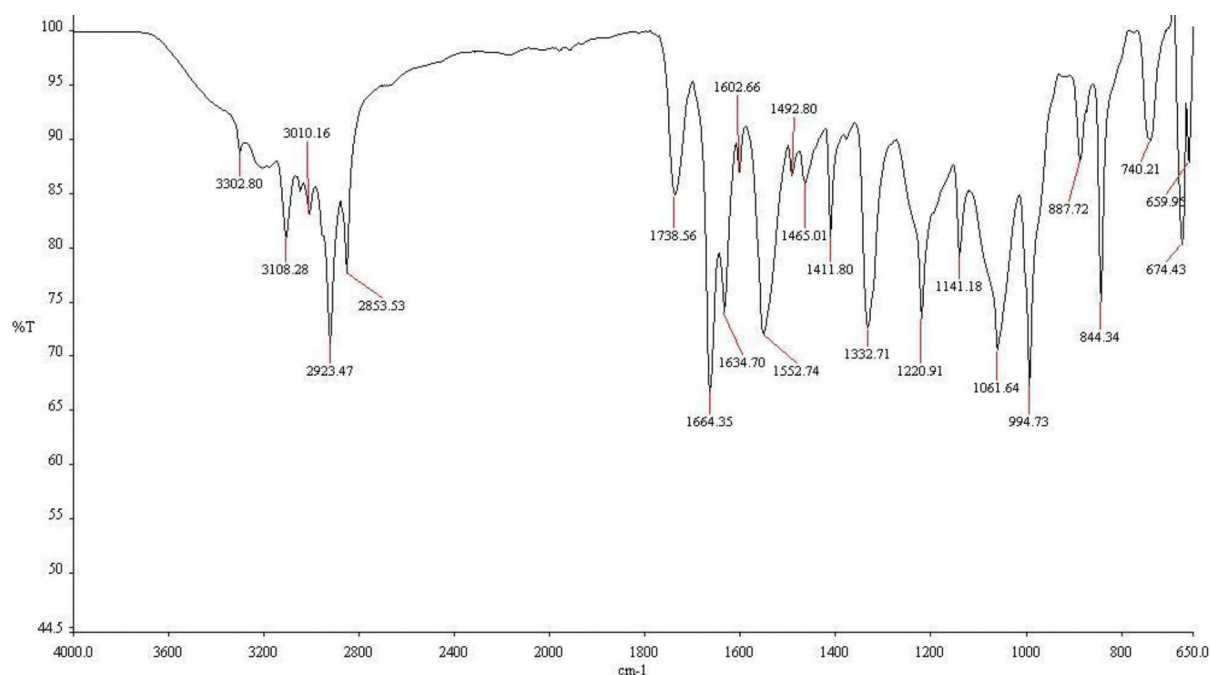


Figure 3.12: FTIR spectrum of the liposomal formulation CL-F1.

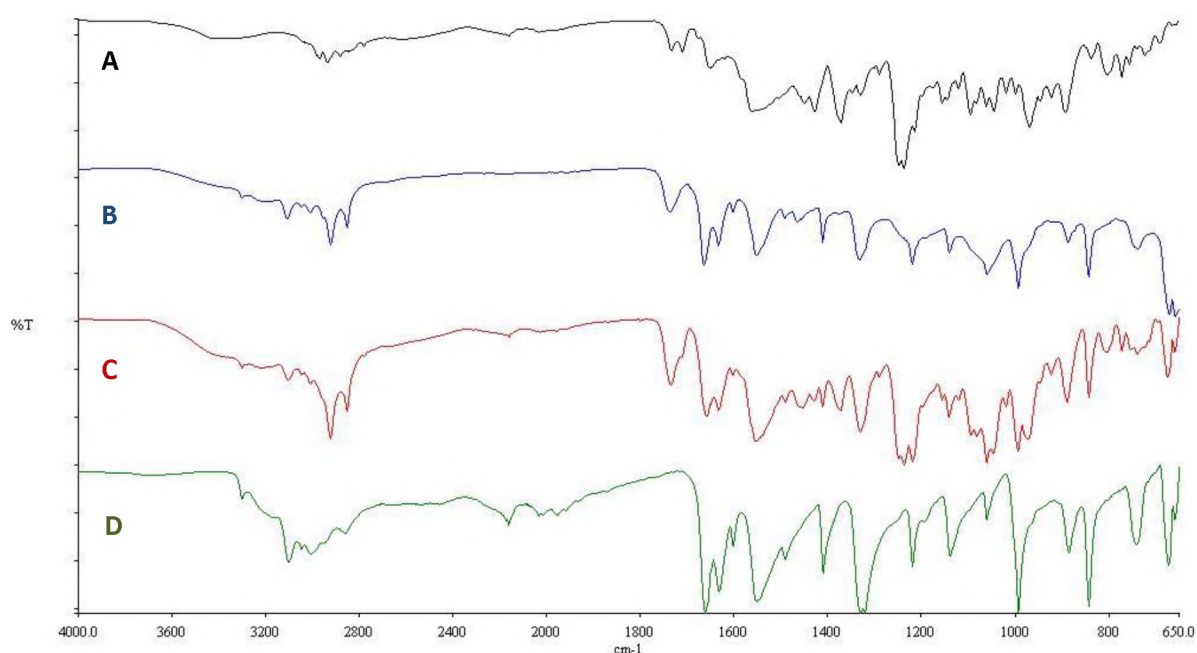


Figure 3.13: FTIR spectra of pure RIF (A); CL-F1 (B); physical mixture of RIF + crude lecithin + INH (C); and pure INH (D).

3.8. Drug release profile

Data from drug content evaluation and release study conducted *in vitro* in simulated physiological conditions (pH 7.4 phosphate buffers maintained at 37 °C) are presented in **Tables 3.7** and **3.8** respectively. The liposomal formulation CL-F1 showed the highest content for the 2 drugs, $9.36 \pm 1.41\%$ for RIF and $49.97 \pm 1.23\%$ for INH.

Table 3.7: Data from drug content determination (n=3).

Formulation	INH content $\pm$ SD (%)	RIF content $\pm$ SD (%)
CL-F1	49.97 ± 0.82	9.36 ± 1.41
CL-F4	49.52 ± 1.23	7.05 ± 0.51
CL-F7	44.51 ± 3.21	6.73 ± 1.23
CL-F10	–	14.23 ± 1.48
CL-F11	51.56 ± 1.66	–

Overall, the release profile of INH was faster than that of RIF either as free drugs or in liposomal formulations. This is in disagreement with the result reported by Gürsoy et al. (2004) who got almost an identical release for the two drugs. The present observation makes sense as INH is smaller, more polar and water soluble than RIF – with respective aqueous solubility of 125 mg/ml versus 1–2 mg/ml (Sosnik et al., 2010). The zero order release kinetics for INH and RIF from the liposomes CL-F1, CL-F4 and CL-F7 appear slower, controlled and sustained as compared to the release patterns of the free drugs (**Figure 3.14**).

Table 3.8: Data from *in vitro* release study for liposomal formulations and free drugs.

Time (hour)	CL-F1- INH (%)	CL-F1- RIF (%)	CL-F4- INH (%)	CL-F4- RIF (%)	CL-F7- INH (%)	CL-F7- RIF (%)	CL-F10 RIF (%)	CL-F11 INH (%)	RIF Free (%)	INH Free (%)
0	0	0	0	0	0	0	0	0	0	0
0,5	43	12	41	18	48	18	10	52	9	60
1	59	16	60	23	62	23	18	62	15	75
1,5	67	21	66	28	63	25	26	70	25	88
2	69	27	69	33	64	29	32	71	29	93
3	70	38	70	39	65	34	42	76	38	97
4	75	39	73	46	66	38	51	77	46	100
5	79	43	75	50	67	43	60	78	54	–
7	82	48	78	53	69	47	67	80	61	–
9	83	49	79	54	70	53	74	81	68	–
12	85	54	81	56	72	57	83	83	80	–

Unlike Patil et al. (2015) who observed a decrease in the release rate of RIF with cholesterol increase, the release kinetic of RIF appears similar for these three formulations, despite the difference in the contents of cholesterol. However, the release profile of INH seems to be slightly affected by cholesterol; with 85%, 81% and 72% of INH released respectively by liposomes CL-F1, CL-F4 and CL-F7 within 12 hours. The release kinetic of INH from these liposomes tends to decrease as the proportion of cholesterol increases in the formulation, probably due to the reduction of membrane permeability as cholesterol strengthens the packing of phospholipid bilayers (Bozzuto and Molinari, 2015; Pattni et al., 2015).

As shown in **Table 3.9**, the Korsmeyer-Peppas model showed the highest values of the Coefficient of determination (R^2), which indicates that diffusion and erosion modes are the main release mechanisms of the encapsulated drugs from liposomes CL-F1 (Costa and Lobo, 2001). Although the dual release showed by CL-F1 was significantly slower than the free drugs (p -value <0.05), there is a huge difference between the amounts of the two drugs released by these liposomes within 12 hours, 85% of INH and 56% of RIF, as compared to other formulations (**Figure 3.15**). This makes CL-F1 a promising dual formulation in terms of pharmacological expectations, as INH is supposed to perform a quick sterilizing action on *M. tuberculosis* while RIF is expected to ensure a slow and permanent bacterial killing (Kumar et al., 2011). Considering the mono-drug loaded liposomes in comparison with their equivalent dual liposomal system, CL-F10 showed a significantly faster RIF release than CL-F1 (p -value <0.05), while the release profile of INH from CL-F11 was almost the same as that of the dual liposomes (**Figure 3.16**). This observation is in close agreement with the

result reported by Gürsoy et al. (2004) who co-encapsulated INH and RIF in liposomes made of dipalmitoylphosphatidylcholine and cholesterol.

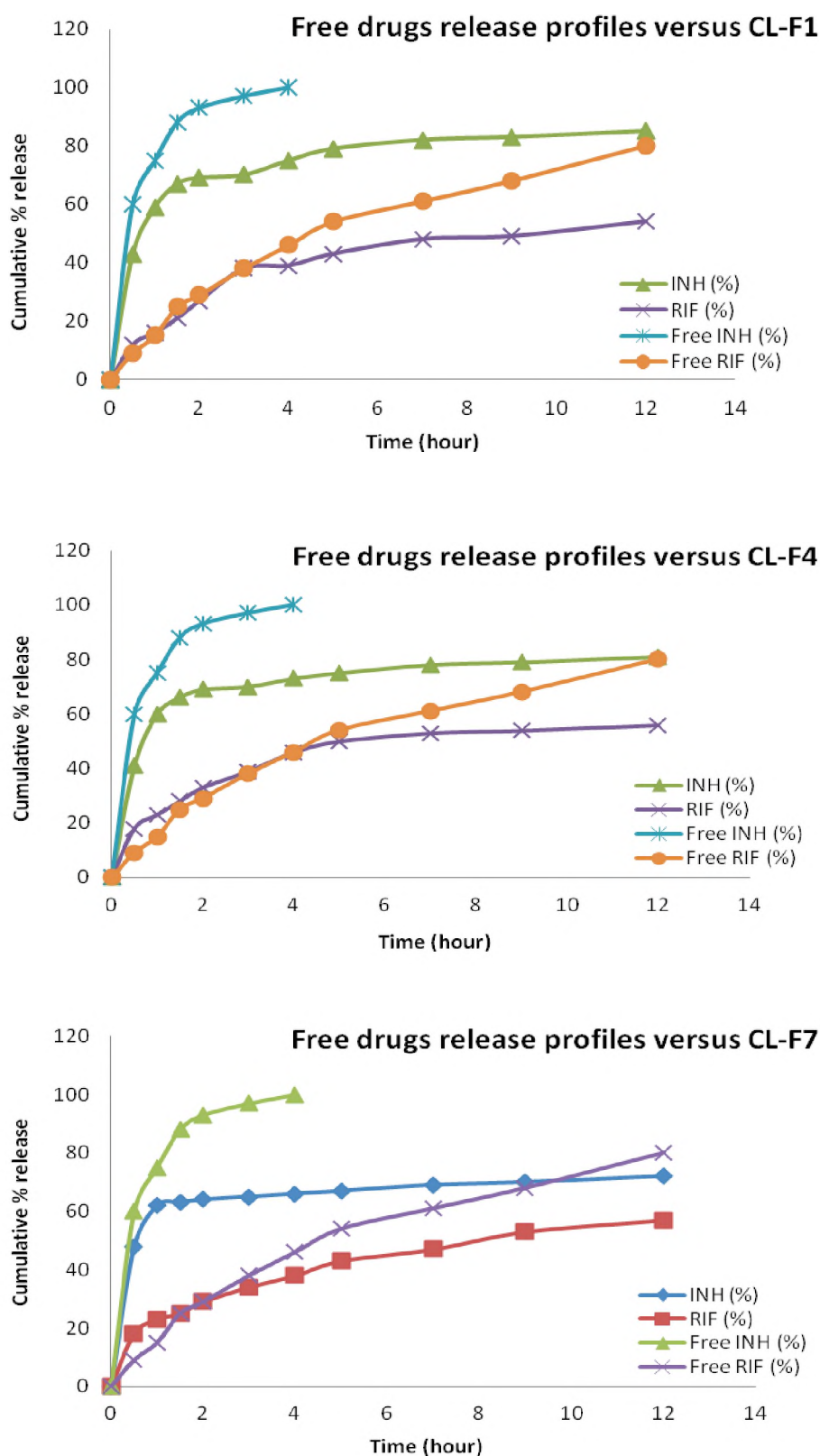


Figure 3.14: Comparison between the zero-order release profiles of the formulations CL-F1 (upper graph), CL-F4 (middle graph), CL-F7 (lower graph) and the free drugs.

Table 3.9: Coefficient of determination (R^2) for the release models of the drugs from CL-F1 liposomes.

R^2	Release model			
	Zero order	First order	Higuchi	Korsmeyer-Pappas
R^2 for INH	0.6782	0.8411	0.8112	0.9596
R^2 for RIF	0.8207	0.8754	0.9232	0.9327

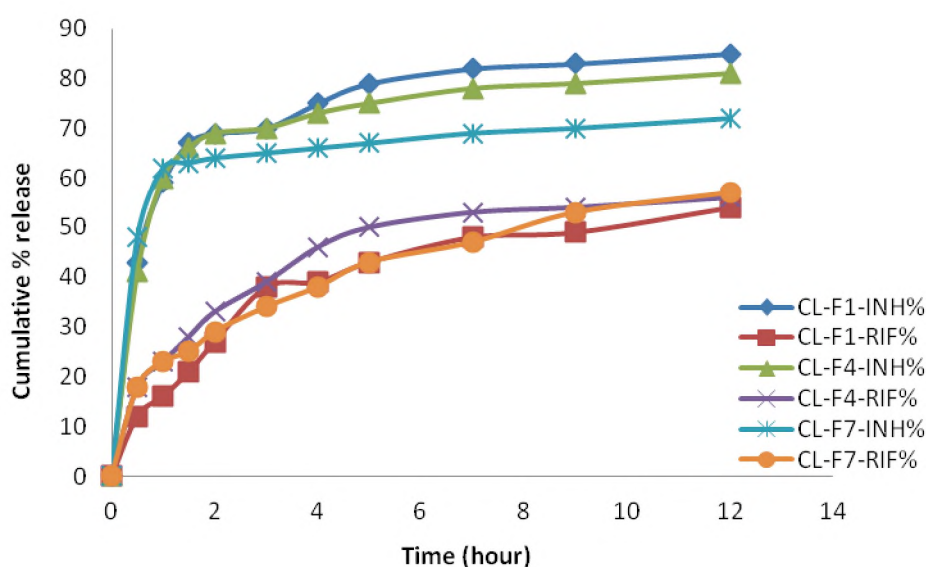


Figure 3.15: Comparison between the zero-order release kinetics of the formulations CL-F1, CL-F4 and CL-F7.

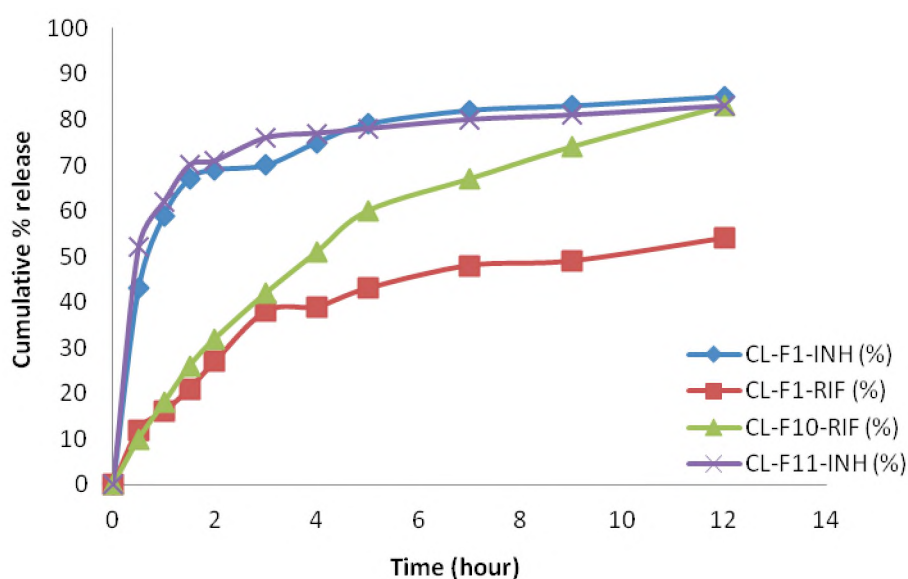


Figure 3.16: Comparison between the zero-order release profiles of the formulations CL-F1, CL-10 and CL-11.

4. CONCLUDING REMARKS

This chapter aimed to encapsulate simultaneously the two major first-line anti-TB drugs in a single liposomal system using crude soybean lecithin (CL). The findings revealed that the liposomes made of CL alone could co-encapsulate INH and RIF more efficiently than those containing cholesterol in the composition. The CL-based liposomes (CL-F1) demonstrated high encapsulation efficiency (%EE) for the two drugs, $59.03 \pm 0.68\%$ for INH and $90.10 \pm 1.15\%$ for RIF. This is surprising considering the %EEs reported by other researchers that have previously discussed simultaneous or separate encapsulation of these drugs in liposomes (Patil et al., 2015; Singh et al., 2015; Manca et al., 2012; Rojanarat et al., 2011; Chimote and Banerjee, 2010; Gürsoy et al., 2004; Justo and Moraes 2003; Deol et al., 1997). In this study, the %EE of INH and RIF in liposomes made of purified soybean lecithin could not reach the higher values achieved by their equivalent CL-based liposomes.

Interestingly, the average particles size of the dual liposomes containing only CL (CL-F1), 1114.00 ± 42.37 nm, was found in between the optimum range for efficient deep lung deposition and macrophage uptake, 1000–3000 nm (El-Sherbiny et al., 2015). The zeta potential of CL-F1, -63.30 ± 2.13 mV, would be promising for the stability of liposomes during the storage (Pattni et al., 2015) as well as their internalization by macrophages (Nagayama et al., 2007). Additionally, the absence of cholesterol in this formulation is worth mentioning. Indeed, cholesterol was reported to interfere with the lung surfactant mimic action of phospholipids, which is essentially needed to cover the depletion of endogenous lung surfactant related to the presence of *Mycobacterium tuberculosis* in alveoli (Chimote and Banerjee, 2010).

The release profiles showed by the dual liposomes CL-F1 in simulated pulmonary release conditions were slower than those of the free drugs were. Nevertheless, the release kinetics of the two drugs from these liposomes seems to be completely different, INH being released quicker than RIF. This makes CL-F1 a promising dual formulation in terms of biological actions, as INH is expected to produce a fast bacterial killing effect while RIF is supposed to supply a slow and permanent sterilizing action (Kumar et al., 2011). However, further investigations on a broad range of process and formulation parameters such as freeze thawing and freeze-drying conditions, the use of cryoprotectants, etc.; would be useful for the development of a liposomal dry power formulation for pulmonary TB therapy.

GENERAL CONCLUSION

The main aim of this study was to develop liposomes suitable for the delivery of anti-tubercular drugs by pulmonary route. The potential of crude soybean lecithin as an alternative to purified phospholipids for liposomal drug delivery was also investigated as part of the research scope. In the course of this work, various liposomal preparations were formulated and evaluated. The liposomes with attractive properties for the intended application were selected for drug encapsulation study. The HPLC methods for the quantification of INH alone and together with RIF were validated for their suitability under actual analytical conditions.

Firstly, INH was encapsulated as a hydrophilic model drug and the prepared drug-loaded liposomes were characterized using various physicochemical techniques. The liposomes made of crude soybean lecithin (CL) showed high encapsulation efficiency of INH ($78.78 \pm 2.45\%$) that has not yet been reported elsewhere. The release kinetics of INH from these liposomes was slower than the profile described by the free drug and the liposomes made of purified lecithin, which besides showed a relatively poor encapsulation efficiency ($20.90 \pm 2.10\%$). Additionally, the mean particle size (813.00 ± 9.21 nm) and zeta potential (-42.80 ± 4.31 mV) of CL-based liposomes seem to be promising for macrophage-targeting pulmonary delivery.

Furthermore, RIF was incorporated as a hydrophobic model drug in the formulation in order to comply with the recommended multidrug-therapy for tuberculosis. The liposomes made of purified soy lecithin could not afford the higher %EE achieved by their equivalent CL-based liposomes. Surprisingly, among several liposomal preparations formulated, the liposomes made of CL alone demonstrated the highest encapsulation efficiency (%EE) for the two drugs. The average particles size and zeta potential of these dual liposomes made of CL, 1114.00 ± 42.37 nm and -63.30 ± 2.13 mV respectively, seem to be attractive features for their shelf stability, deep lung deposition and macrophage uptake (Pattni et al., 2015; El-Sherbiny et al., 2015; Nagayama et al., 2007). In addition, CL-based liposomes showed a promising dual release profile that might closely agree with the pharmacological expectation regarding the mechanism of action of INH and RIF. These two drugs were found to be released by diffusion and erosion modes as the main release mechanisms.

To the best of our knowledge, this work appears to be the first of its kind reporting the feasibility of using crude soybean lecithin to encapsulate efficiently INH alone or in combination with RIF for liposomal delivery. The anti-TB drug loaded liposomes prepared in this study demonstrated attractive features that would be useful for improving the bioavailability of anti-TB drugs at the site of interest.

The present findings suggest the possibility of using a cheap naturally occurring phospholipid mixture, crude soybean lecithin, as an alternative to synthetic or purified natural lipids in liposomal drug delivery. This would inspire other researchers to investigate on the liposomal delivery of various other existing and emerging drugs using any types of lipid mixtures readily available, such as crude rice lecithin, egg lecithin, etc. The use of such cost effective lipid materials in drug delivery would revolutionize the costly formulation status of therapeutic liposomes.

Nevertheless, further studies aiming at formulating liposomal dosage forms for inhalation would be useful for *in vitro* and *in vivo* biological evaluations of these liposomes made of crude soybean lecithin. Other investigations on grafting active targeting-ligands for macrophage receptors would add more pharmacological features to the formulations developed. Additionally, the incorporation of materials sensitive to the changes in pH or redox potential would enhance the bioavailability of anti-TB agents under intra-macrophage level.

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Zur der Impetigines (1839) Auszug aus einer brieflichen Mitteilung an den Herausgeber.
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