In pharmaceutical research, one of the major goals has been to synthesize or discover new chemical entities with desirable therapeutic properties but without undesirable side effects. However, the development of new drug molecules seldom fulfils the medical expectations and also search of new molecules is demanded heavily in terms of money, labor and time. Over and above all, the molecules often express their unwanted effect after a very long latent period, hence not time tested (Dube et al., 2012a). Thus, in the light of these problems with the conventional research, scientists have always been in the search of alternative strategies to strengthen up the existing therapeutic armory. Amongst many approaches, the one which has drawn a great deal of attention is “Neo Delivery Devices” wherein the therapeutic molecules are delivered at the site of action in a desired manner and in a right frame of environment, simulated by the carrier system. In this way, the same existing, old drugs can find a renewed potential in its intelligently designed delivery–vehicle that provides a new lease to life with enhanced effectiveness and minimized side effects (Dube and Vyas, 2010).

The use of non-viral nanocolloidal systems has gained increasing interest within therapeutics, revealing exciting prospects. Due to their low toxicity in comparison with viral systems, they are good candidates for targeting tissue and cells with different compounds. For this purpose, drugs, peptides and nucleic acids of poor stability have been combined with polymers and lipids to obtain very fine, sub-micron systems that have the ability to interact with the target cells and be internalized by them (Vyas and Khar, 2002).

1.1. TUBERCULOSIS

Tuberculosis (TB) is primarily a disease of the respiratory system and is spread through inhalation by healthy individuals of *M. tuberculosis* (*Mt*) containing droplets released into the environment by the cough of tuberculosis patients. *Mt* is a highly pathogenic
bacterium - it has been estimated that inhalation of a droplet containing as few as 1-10 bacteria is sufficient to initiate and establish an infection of the airways of a healthy individual.

TB is a leading killer of young adults worldwide. In the last two decades, TB has gone from being a forgotten disease to a modern and recrudescent pathology. Even though the infection afflicts mainly South-East Asia, Western Pacific regions and Africa, many worldwide efforts have gathered to fight this worrying disease. TB has resurrected with a new face and the global scourge of multi-drug resistant TB (MDR TB) is reaching epidemic proportions. Globally, there were estimated 8.8 million incidences (new) of TB in 2010 including 1.1 million cases among people with HIV. In 2010, 1.4 million people died from TB, including 350,000 people with HIV, equaling 3,800 deaths a day. Most of the cases occurred in the South-East Asia Region (55%) and the African Region (30%). The five countries with the largest numbers of cases in 2008 included India (1.6–2.4 million), China (1.0–1.6 million), South Africa (0.38–0.57 million), Nigeria (0.37–0.55 million) and Indonesia (0.34–0.52 million). Of 9.4 million new TB cases reported in 2008, an estimated 1.4 million (15%) were HIV positive; where 78% of these HIV-positive cases were in the African Region and 13% were in the South-East Asia Region (WHO, 2011).

1.1.1. *Mtb* - Causative Agent of TB

The genus *Mycobacterium* comprises mostly soil dwelling saprophytes, and only a few members of the genus have evolved to adopt a pathogenic lifestyle. Pathogenic mycobacteria cause diseases of diverse nature and varying severity. Tuberculosis is caused by members of the *Mtb* complex that consists of *Mtb*, causative agent of tuberculosis in humans, *M. africanum* that causes tuberculosis in parts of Africa, *M. bovis*, which causes tuberculosis in mammals including cattle and humans, *M. microti*
that infects voles but is avirulent in humans and mice and *M. canettii*, whose infection is rare (Cosma *et al.*, 2003). Though humans are the only natural hosts for *Mtb*, experimental animal models exists, the most commonly used being the mouse (Orme, 2003).

Other members of pathogenic mycobacteria include *M. leprae*, which are the causative agent of the human disease leprosy, *M. marinum*, which causes granulomatous infection in frogs and fish as well as skin lesions in humans (called swimmer’s granulomas) and *M. ulcerans*, which cause Buruli ulcers. Finally, members of *M. avium* complex (*M. avium* subspecies avium, *paratuberculosis* and *silvaticum* and *M. intracellulare*) can occur as opportunistic pathogens especially in immuno-depressed patients (Cosma *et al.*, 2003).

The genomes of several members of pathogenic mycobacteria have been sequenced. *Mtb* was among the first organism whose complete genome sequence was known (Cole *et al.*, 1998). Sequencing of the *M. leprae* genome showed massive gene decay as compared with *Mtb*, which is indicated by the genome sizes and the predicted gene numbers of the two species; while the genome size of *Mtb* is 4.4 Mb encoding 4000 genes, the genome size of *M. leprae* spans 3.27 Mb coding for only 1600 genes. Entire metabolic pathways are purged from the *M. leprae* genome (Cole *et al.*, 2001), and it has been proposed that *M. leprae* has retained only essential genes and pathways in its adaptation to a pathogenic lifestyle. Genes conserved between the two species are hence considered particularly important for pathogenicity and virulence.

1.1.2. Route and Site of Infection

Man is the primary host for *Mtb*. Infection of a host with *Mtb* is initiated following the inhalation of droplets (aerosols) containing a small number of bacilli (Kaufmann, 2001). The bacilli spread from the site of initial infection in the lung through the lymphatics or
blood to other parts of the body; the apex of the lung and the regional lymph node being favoured sites. Extrapulmonary TB of the pleura, lymphatics, bone, genito-urinary system, meninges, peritoneum, or skin occurs in about 15% of TB patients.

Once in the lung, bacilli are internalized through phagocytosis by the resident macrophages of the lung, the alveolar macrophages; the first event in the host-pathogen relationship that decides outcome of infection. Alveolar macrophages activated by the appropriate stimuli can effectively transfer the phagocyted *Mtb* to the destructive environment of lysosomes, but some bacilli are able to escape lysosomal delivery and survive within the macrophage (Armstrong and Hart, 1975; Kaufmann, 2001; Russell, 2001). Infected macrophages can then either remain in the lung or are disseminated to other organs in the body. However, only a minority (almost 10% of infected people develop TB, because in most healthy individuals, the immune defence system is sufficient to keep *Mtb* in check such that disease cannot develop.

**1.2. MACROPHAGES - THE TARGET CELLS**

*Mtb* invades and replicates in macrophages, cells of the host innate defense system that is designed to clear pathogenic microorganisms. The ability of pathogenic mycobacteria to adapt to the hostile environment of macrophage has been instrumental in its success as a pathogen. Mycobacteria interfere with host trafficking pathways by modulating events in endosomal/phagosomal maturation pathway to create a protected niche for itself, the mycobacterial phagosome (Houben *et al*., 2006). This phagosome, containing living mycobacteria, while connected to the endocytic pathway, does not fuse with or mature into lysosomes (Hart and Young, 1991). The non-fusogenicity of mycobacterial phagosomes is believed to be a major factor in the capacity of pathogenic mycobacteria to survive within the hostile environment of the macrophages (Nguyen and Pieters, 2005; Vergne *et al*., 2004). Before going in to the details of the macrophage- *Mtb* interactions let us first discuss the phagocytic cells.
Phagocytic cells represent for an essential component of the immune system and their main function being to ingest and destroy microorganisms. There are different types of phagocytic cells. Monocytes are a type of phagocytic cell which are found in the bloodstream. When monocytes leave the circulation and penetrate tissues, they change shape and become macrophages. Macrophages are the major differentiating cell of mononuclear phagocyte system, which comprises bone marrow monoblast and promonoblast, peripheral monocytes and tissue macrophages. The precursors of macrophages are monocytes, promonocytes, and monoblasts. All of these cells belong to a common progenitor; called the colony-forming unit, granulocyte-macrophages. Monoblasts, the least mature cell of the mononuclear phagocyte system, firstly differentiate into monocytes and remain in the bone marrow for 24 h and then they enter the peripheral blood. From the peripheral blood, monocytes migrate to extra vascular tissue where they differentiate into macrophages (Aauger and Ross, 1992). Macrophages colonize the liver (kupffer cells), lungs (alveolar interstitial macrophages), spleen, lymph nodes, thymus, gut, marrow, brain, connective tissue and serous cavities.

Macrophages play a critically prominent role in host defence against many infectious agents, including bacteria, viruses, protozoa and parasites. Macrophages are migrated to an infected focus following attraction by a variety of substances, which include bacterial components and endotoxins, complement components, immune complexes and collagen fragments. Once they are at the infected focus, the macrophages may phagocytose and kill the infectious agents by a variety of mechanisms. By taking protein antigens and generating immunogenic fragments from them, macrophages also play a significant role in inducing and regulating the immune response. Owing to their appreciation and affinity for receptor mediated disposition of the endogenous ligands and macrophage mediated activation and immunological consequences, macrophages are well investigated in the field of immunology, biotechnology and drug delivery.
1.2.1. Phagocytosis by Macrophages

Phagocytosis in classical sense is the engulfment of the endogenous and exogenous particulate materials, such as bacteria, erythrocytes, latex beads and colloidal particles. Multiple attachments of particle associated ligands with membrane receptors is an essential stimulus for phagocytic capture of particles. It is often performed by the phagocytic cells of the reticuloendothelial system (RES) including the Kupffer cells of the hepatic sinusoids, the tissue fixed macrophages (histocytes) and the blood macrophages or monocytes. The process of phagocytosis involves sequential steps of:

- **Recognition/adhesion** (chemotaxis and adherence mediated by coating of blood components, mainly opsonins and high density lipoproteins);

- **Ingestion** (attachment and engulfment of the particle to the macrophage cells of the RES); and

- **Digestion** (whereby the particles are transferred to phagosome, phagolysosome and finally to digestive vacuoles).

Phagocytosis by macrophages is governed and regulated by certain factors, which prevent the engulfing of endogenous structures and at the same time selective phagocytosis of only foreign non-self particles takes place. Extent of particle sequesteratation and clearance by macrophages is determined primarily by the physicochemical nature of the surface, however, other factors, such as nature of the particle matrix, particle stability and physiological state of the species can be important. Macrophages possess high phagocytic propensity towards rough surfaces, which are typical characteristic of dead tissues or particles of foreign origin. Endogenous structures, however escape the phagocytosis because of their smooth surfaces and protective protein coats.
1.2.2. Receptors Associated with Macrophages

Free drug enters the cell interior via transmembrane diffusive transport or non-specific adsorptive pinocytosis, while cellular uptake of a drug-carrier composite is mostly restricted to receptor mediated endocytosis. When transported by diffusion, the drug reaches the cytoplasm of the cell, whereas all pinocytic processes are lysosomotropic. Cellular uptake of many endogenous and exogenous ligands takes place via receptor mediated endocytosis. Receptors are membrane-associated proteins having specific affinity for a variety of molecules such as polypeptides, polysaccharides, glycolipids or proteins. The latter smaller molecules, which bind to the receptors specifically, are referred to as ligands. The macrophages express several specific receptors for a spectrum of ligands. The ligands known to have specific receptor could be utilized in controlled and targeted drug delivery to the macrophages.

The receptors commonly present or expressed under stimulated conditions on the macrophage; and able to negotiate endocytosis upon interaction with ligands are given in Table 1.1 and illustrated in Fig 1.1. Macrophages do not express clonal idioptypic receptors like lymphocytes but they express receptor molecules, which are selective for a category of ligands e.g., sugar specific recognition of glycoconjugates or the common Fc fragments of IgG subclasses.

The phagocytosis of particulates by macrophages in culture is thought to occur via the adsorption of plasma protein by a process known as opsonization. The opsonized particles adhere to the phagocytic cell membrane and are subsequently internalised by phagocytes. The surface of macrophages bears over thirty receptors, including those corresponding to the Fc portion of immunoglobulins and the C3b component of the complement system. Opsonins mediate the onset of phagocytosis by their ability to bind to the particle surface and to the target receptors on the phagocytes. Since the Fc region
is common to all immunoglobulins, regardless of antigenic specificity, IgG can act as an effective bridge between the macrophage and the foreign materials that are to be phagocytosed (Vyas, 2000).

Macrophages also express heterogeneous receptors (Table 1.1) like the distinct receptor molecules for different isotypes of IgG as well as the receptors, which are capable of interacting with several ligands. Besides phagocytosis and pinocytosis, macrophage receptors play an important role in growth and differentiation, secretion, metabolic responses, cell activation, macrophage migration and adhesive interaction with other cells and micro-organisms.

1.2.2.1. Scavenger Receptors

The scavenger receptors were functionally identified and defined by Brown and Goldstein for their ability to bind and internalize modified low density lipoproteins (mLDLs) such as oxidized LDL (Ox-LDL) and acetylated LDL (Ac-LDL), but not native LDL (Goldstein et al., 1979). These multifunctional receptors are involved in various cellular functions ranging from adhesion, endocytosis and phagocytosis to antigen presentation. The molecular and cell biology of the different classes of scavenger receptors and their active domain’s structures and role in lipid metabolism have been reviewed extensively (Krieger, 1997; Murphy et al., 2005).

1.2.2.2. CD14

CD14 is a glycosylphosphatidylinositol (GPI) - linked glycoprotein that is highly expressed on macrophages and monocytes (Dziarski, 2003). The ligand binding site is made up of eight to ten leucine rich glycoprotein repeats. Ligand recognition by CD14 results in macrophage activation and inflammatory cytokine production. However, the receptor is not a cell-activating receptor by itself; rather it functions as a co-receptor with
TLR4 in response to LPS, and enhances cell activation of TLR2 in response to peptidoglycan.

### 1.2.2.3. C-type Lectins

The C-type lectin receptors recognise carbohydrates on cell surfaces, circulating proteins and also on pathogens (McGreal et al., 2004). Classical C-type lectin receptors contain a hydrophobic fold termed as carbohydrate-recognition domain (CRD), which binds carbohydrate molecules in calcium (Ca\(^{2+}\))-dependent manner. However, it has now come to be known that many members of the C-type lectin family contain CRDs which do not bind Ca\(^{2+}\) and these receptors also interact with non-carbohydrate ligands. Therefore, the term C-type lectin-like domain (CTLD) is used to denote the common fold within this protein family without referring to functional similarities (Weis et al., 1998).

Membrane-bound C-type lectin receptors recognise a diverse range of ligands and can be divided into three groups based on their CTLD: (1) C-type lectins containing a single CRD; (2) C type lectins containing multiple CRDs; and (3) NK-like C-type lectin-like (NKCL) receptors, which have a single CRD. The CTLDs of the NKCL receptors lack the residues involved in Ca\(^{2+}\) binding, setting them apart from the other two groups. Members of the C type lectins with a single CRD are typically type II membrane receptors which include DC-SIGN (‘dendritic-cell-specific intercellular-adhesion molecule-3-grabbing non-integrin’) and Dectin-2.

C-type lectins containing multiple CRDs are type I membrane receptors and include the mannose receptor, Endo180, and the related DEC-205 and phospholipase A2 receptor. The type II membrane receptors also termed NKCL receptors include Dectin-1 and CD69. These receptors typically possess a single extracellular carbohydrate-binding domain (CTLD), a stalk region, a transmembrane domain, and a cytoplasmic tail with or without signalling motifs.
Table 1.1: Various macrophage receptors which trigger phagocytosis

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Ligands</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) FcR1</td>
<td>Monomeric and complexed IgG</td>
<td>Monocytes and macrophages</td>
</tr>
<tr>
<td>(ii) FcR2</td>
<td>Complexed IgG</td>
<td>Monocytes, macrophages, Granulocytes</td>
</tr>
<tr>
<td>(iii) FcR3</td>
<td>Complexed IgG</td>
<td>Mature macrophages, Natural Killer-cells, granulocytes</td>
</tr>
<tr>
<td>Complement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) CR1</td>
<td>C3b</td>
<td>Macrophages</td>
</tr>
<tr>
<td>(ii) CR2</td>
<td>C3b1</td>
<td>Macrophages</td>
</tr>
<tr>
<td>(iii) CR4</td>
<td>C3b1</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Mannose/Fucose</td>
<td>Mannose/ fucose residues</td>
<td>Mature macrophages and hepatic endothelium</td>
</tr>
<tr>
<td>Glucan</td>
<td>Glucan residues</td>
<td>Mature macrophages</td>
</tr>
<tr>
<td>α-Macroglobulin</td>
<td>Protease complex</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Scavenger</td>
<td>Modified lipo-proteins, polysaccharides, polynucleotides, phospholipids, bacterial lipopolysaccharides</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Galactosyl</td>
<td>Galactose</td>
<td>Kupffer cells</td>
</tr>
<tr>
<td>WGA receptor</td>
<td>Wheat germ agglutinins</td>
<td>Macrophages</td>
</tr>
</tbody>
</table>

1.2.2.4. Integrins

Integrins are heterodimeric plasma membrane receptors. Their main function is to attach the cells to the extracellular matrix (ECM), as well as signal transduction from the ECM to the cell. Two of the complement receptors – CR3 (CD11b/CD18) and CR4 (CD11c/CD18) – belong to this receptor family and are termed b2 integrins as they have the same b subunit (Arnaout et al., 2005). CR3 and CR4 are found not only on
macrophages but also on neutrophils, eosinophils, basophils, monocytes/ macrophages, natural killer cells, microglial cells and platelets. Most ligands for CR3 are recognized through a chain, which consists of three domains, namely an extracellular, transmembrane and cytoplasmic domain.

Fig. 1.1: Various receptors associated with macrophages
1.2.2.5. Toll-like Receptors

The TLRs are expressed on the surface of many cells, including epithelial cells, monocytes and dendritic cells. They are generally type I transmembrane proteins with an extracellular domain that contains leucine-rich repeats (LRRs) and a cytoplasmic portion with a homology to the interleukin 1 receptor family (IL-1R) (Stenger and Modlin, 2002). The cytoplasmic portion interacts with adaptor molecules MyD88 (myeloid differentiation primary response protein 88), TRIF [Toll-interleukin 1 receptor (TIR)-domain-containing adaptor inducing IFN-b], TRAM (Toll-like receptor adaptor molecule) and TIRAP/Mal (TIR-domain containing adaptor protein) and the adaptor molecules vary between different TLRs (O’Neill, 2006).

1.2.3. Mechanism for Uptake

Clathrin dependent endocytosis, an energy dependent process, is the best characterized phenomenon of cellular internalization. Initial uptake of ligands into coated vesicles is followed by fusion with early, tubulovesicular endosomes near the plasma membrane.

There are also clathrin-independent mechanisms for endocytosis of which caveolae-mediated endocytosis has been widely investigated. Caveolae are small, coated invaginations of plasma membrane that are rich in cholesterol and glycosphingolipids. Caveolae do not separate from the plasma membrane while unloading their cargo and is advantageous for drug delivery because it avoids acidic compartments (endosomal/lysosomal pathways). These uncoated vesicles sometimes deliver their contents to endosomes and lysosomes, process being termed as potocytosis. Macropinocytosis is an actin-dependent endocytotic pathway where irregular sized and shaped vesicles are formed. This is a non-selective phenomenon involved in the uptake of large volumes of fluid. The vesicles (macropinosomes) do not fuse with endosomes or lysosomes despite fusing with each other; however, sometimes macromolecules
internalized in macropinosomes have been found to be delivered to lysosomes (Rawat et al., 2007).

Following endocytosis, macromolecules are initially entrapped in endosomes, an acidic compartment (pH 5.5–6.5) and subsequently transferred to the lysosomes. This process occurs by content mixing between the late endosome and lysosome as a result of ‘kiss-and-run’ events and/or direct fusion between two organelles. The endosomal compartment is a complex structure of tubules and vesicles which mediates the sorting of the internalized cargo to the subsequent compartment through vesicle fusion depending on the nature of the receptor. The acidic environment is responsible for the dissociation of most of the ligands from their receptors. Lysosomes, similar to the endosome, contain a greater number of hydrolytic enzymes and are acidic in nature. These are membrane bound vesicles and constitute a barrier to the transport of macromolecules and delivery system (Bulmus, 2005).

Receptor-ligand complexes after internalization appear to follow multiple pathways, which vary depending on the signal carried by the receptor. Therefore, depending on the nature of the receptor, the receptor-mediated endocytosis (Fig. 1.2) is classified into one of the following four types:

- Receptors are recycled and ligand is degraded in the lysosomes; eg., low density lipoprotein, mannose or galactose terminated oligosaccharides/glycoproteins.
- Both receptors and ligands are recycled; eg., transferrin, major histocompatibility antigens.
- Both receptors and ligands are degraded; eg., Fc receptors, epidermal growth factor.
- Receptors degraded and ligands are transported across the cell; e.g., immunoglobulin A and immunoglobulin M.
1.2.4. Intracellular Trafficking of Endocytosed Ligands

After endocytosis of the receptor-ligand complex, the transport of materials from one intracellular compartment to another occurs through vesicular budding and fusion. Vesicles containing drug cargo bud off from the donor compartment and deliver their contents to the acceptor compartment by fusion with the latter. A series of small GTP binding proteins regulate membrane trafficking events by altering between two conformations in a nucleotide-dependent fashion where GTP bound form turns the protein “on” while hydrolysis of the GTP to GDP turns the protein “off”. These activities...
are regulated by a series of generic and compartment-specific proteins which are specifically localized on distinct intracellular compartments and presumably control a specific transport step (Balch, 1990; Rothman and Sollner, 1997). Among the monomeric small GTPases, the Rab family of Ras related proteins is well characterized.

The proteins of the family are regulators of intracellular trafficking during endocytosis and secretion. Rab proteins are specifically localized into the cytoplasmic surface of the intracellular compartments. Internalized receptor and ligand first enter the peripheral sorting endosome where the membrane proteins destined to the degradation or trans Golgi network (TGN) are sorted out from membrane proteins and targeted and subjected to the recycling back to the plasma membrane. A substantial body of evidence indicates that Rab 5 regulates endocytosis from the plasma membrane to the early endosomes and is also involved in homotypic fusion between the early endosomes. Rab 4 appears to control the recycling from the early endosomes to the plasma membrane.

Recent studies indicate that Rab 11 regulates the recycling through the perinuclear endosomes. Rab 7 and Rab 9, and perhaps others still to be identified, are associated with the late endosomal compartments. Rab 9 has been shown to regulate the traffic from the late endosome to TGN while Rab 7 regulates the traffic from the early to late endosomes/lysosome (Lombardi et al., 1993). It has also been shown that Rab 7 regulates the transport between the late endosome to a lysosome-like compartment. However, the exact relationship among all these factors in the overall mechanism of intracellular trafficking during endocytosis is largely unknown.

A current model suggests that Rab proteins are predominantly found on cellular membranes but a significant fraction is also present in the cytosol as a complex with a protein called guanine nucleotide dissociation inhibitor (GDI). GDI presents the complex to the specific organelles through an interaction catalyzed by GDI displacement factor
(GDF) in conjunction with guanine nucleotide exchange factor (GEF). GTP-bound Rab proteins are recruited onto nascent transport vesicles and catalyze the association of V SNARE [soluble NSF attachment protein (SNAP) receptor on vesicle] with T SNARE (SNAP receptor on target) to accomplish docking of the vesicles. Each V SNARE and T SNARE complex binds between 3 to 6 SNAP proteins and then NSF binds to this complex. Subsequently, NSF hydrolyzes the ATP and uses the energy to disrupt the docking complex and releases the SNAP protein. After the fusion GTPase activating proteins (GAP) increase the GTPase rate of the Rab protein, converting it into its GDP-bound conformation. Unoccupied GDI then retrieves the Rab protein for another round of vesicular transport. This is a generalized model but most of the interacting proteins for a particular Rab have yet to be identified.

1.2.5. Macrophage-Mycobacterium Interactions and the Role of Macrophage in Host Response

When a phagocytic cell encounters a pathogen, a series of events result in the internalization, intracellular trafficking along the phagocytic pathway and the subsequent delivery of the pathogen to lysosomes, leading to its degradation. The distinct steps in this pathway include binding of the bacteria to various receptors, invagination of the host cell membrane around the pathogen leading to formation of phagosome, scission of the phagosome and its maturation along the endocytic/phagocytic pathway finally resulting in fusion with or maturation into lysosomes (Booth et al., 2001).

Phagocytosis of \textit{M. tuberculosis} by macrophages proceeds through a series of membrane invagination, budding, and fusion events, resulting in the formation of the phagosome (Aderem and Underhill, 1999). Internalized material is further distributed within the cell through a series of vesicle trafficking events delivering the material to the antigen processing and presentation pathway. The final destination of both the
phagosomal and endosomal cargo is the lysosome, where extensive degradation of the cargo occurs thus, resulting in the clearance of potentially harmful material and subsequent destruction of pathogens. Pathogenic mycobacteria have involved mechanisms to interfere with both (glyco)lipid and protein-mediated mechanisms that regulate the transfer of cargo to lysosomal organelles for destruction.

Complement receptors (CR1, CR2, CR3 and CR4), mannose receptors (MR), dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN), and Fc receptors play an important role in binding of the organisms to the phagocytes (Ernst, 1998). The ability of multiple receptor molecules to internalize \textit{Mtb} is likely related to the complex structure of the cell surface of \textit{Mtb} (Brennan and Nikaido, 1995). The interaction between MR on phagocytic cells and mycobacteria seems to be mediated through the mycobacterial surface glycoprotein lipoarabinomannan (LAM). Prostaglandin E2 (PGE2) and interleukin (IL)-4, a Th2-type cytokine, upregulate CR and MR receptor expression and function, and interferon-\(\gamma\) (IFN-\(\gamma\)) decreases the receptor expression, resulting in diminished ability of the mycobacteria to adhere to macrophages. There is also a role for surfactant protein receptors, CD14 receptor and the scavenger receptors in mediating bacterial binding.

In a majority of infected individuals, a certain number of macrophages exist that harbour viable mycobacteria, yet most infected individuals do not develop disease. This raises the question of how these bacilli are contained by the host immune system. \textit{Mtb} residing within macrophages is kept in check within structures termed ‘Granulomas’ (Cosma \textit{et al.}, 2003; Flynn and Chan, 2003). Although the precise biology of granulomas remain only partially understood, it is believed that granulomas are structured clusters containing \textit{Mtb} infected macrophages in the centre, surrounded by different types of
immune cells, in particular macrophages and T lymphocytes (Gordon et al., 1994). Fig. 1.3 illustrates the formation of granuloma.

Both non-activated and activated macrophages coexist within granulomas, with the activated macrophages processing and presenting mycobacterial antigens to the surrounding T lymphocytes (Chan and Flynn, 2004). Following presentation of mycobacterial antigens, the T cells become activated through the triggering of T cell receptors (Kaufmann, 1993). Activated T cells secrete cytokines and chemokines keeping the macrophages in an activated state and ensuring the recruitment of other immune cells to the site of infection. *M. tuberculosis* may exist as actively dividing bacilli or in a so-called “dormant” state. Also, it cannot be excluded that both actively dividing and dormant bacilli can occur within the same infected individual depending on the stage of the disease (Dheda et al., 2005). Regardless, *M. tuberculosis* can persist for a long time, even up to the lifetime of the host (Young et al., 2002), within these structures, and as long as host immunity (in the form of activated macrophages and functional T cells) is effective, there is usually no adverse effect of the *M. tuberculosis* on the host’s health (Saunders and Britton, 2007).

Thus, the granuloma structure likely represents a balance between a potentially dangerous pathogen and the host immune system. The delicacy of this balance is illustrated by the observation that any deterioration of host immunity results in a potentially life-threatening condition of individual harbouring live *M. tuberculosis* (Bartlett, 2007).

**1.2.6. Phagolysosome Fusion**

Phagocytosed microorganisms are subject to degradation by intralysosomal acidic hydrolases upon phagolysosome fusion. This highly regulated event constitutes a significant antimicrobial mechanism of phagocytes. Hart and co-workers hypothesized that prevention of phagolysosomal fusion is a mechanism by which *M. tuberculosis* survives inside macrophages (Hart et al., 1972). It has been reported that mycobacterial sulphatides,
derivatives of multiacylated trehalose 2-sulphate, have the ability to inhibit phagolysosomal fusion. In vitro studies demonstrated that *Mtb* generates copious amounts of ammonia in cultures, which is thought to be responsible for the inhibitory effect (Gordon *et al*., 1980).

*Mtb* glycolipids can interfere with phagosomelysosome fusion through blocking a normal host trafficking event that is regulated by phosphatidylinositol 3-phosphate (PI3P). Locally generated by phosphatidylinositol 3-kinase (PI3 kinase) on early endosomal and phagosomal membranes, PIP3 is believed to present a docking site for several proteins involved in the maturation of phagosomes into lysosomes, such as the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and the early endosomal autoantigen 1 (EEA1) (Birkeland and Stenmark, 2004; Itoh and Takenawa, 2002). Thus, while in uninfected cells, the generation of PI3P regulates the delivery of phagocytosed cargo to lysosomes, *Mtb* interferes with this trafficking event by actively preventing PI3P accumulation on phagosomal membranes (Fratti *et al*., 2003). SapM is released within the host cell cytosol upon infection, where it hydrolize PIP3 on phagosomal membranes thus preventing its accumulation on phagosomes. PknG is the only soluble kinase maintained in the genome of all pathogenic mycobacteria.

The kinase activity of PknG is essential for the prevention of phagosome-lysosome fusion, since overexpression of a dominant negative mutant form of PknG results in lysosomal delivery of the bacteria (Walburger *et al*., 2004). TACO (for Tryptophan aspartate containing coat protein, now referred to as coronin 1, is an important host factor that specifically prevents the lysosomal delivery and death of mycobacteria inside macrophages (Gatfield *et al*., 2005). When macrophages are infected with mycobacteria, they respond with a sustained calcium flux that is dependent of the presence of coronin 1. The coronin 1-dependent activation of calcineurin is required for
blocking phagosome-lysosome fusion (Fig. 1.4). Transfer of \textit{M. tuberculosis} to lysosomes is blocked almost exclusively in nonactivated macrophages. Following stimulation by cytokines (interferon-\(\gamma\) and TNF-\(\alpha\) are probably the most important) macrophages become activated and bacilli are rapidly transferred to lysosomes and are destroyed by bactericidal activities such as the generation of reactive oxygen and nitrogen, which are also up-regulated upon macrophages activation.

1.3. DRUG THERAPY AND ITS CURRENT LIMITATIONS

Since the control measures for TB such as Bacillus Calmette-Guérin (BCG) vaccination and chemoprophylaxis appear to be unsatisfactory, the main avenue for its control is case finding and treatment. The goals of treatment are to ensure cure without relapse, to prevent death, to impede transmission, and to prevent the emergence of drug resistance. Long-term treatment with a combination of drugs is required. Various drugs which are used in the treatment of TB are tabulated in Table 1.2. The current recommended TB chemotherapy, also known as DOTS (directly observed treatment, short-course), consists of a 6-month therapy of 4 co-administered drugs. As suggested by WHO, treatment of TB and drug resistant cases requires multi-drug therapy, comprising:

- An initial intensive phase of rifampicin (RIF), isoniazid (INH), pyrazinamide (PYZ), and ethambutol (ETB) daily for 2 months.
- A continuation phase of RIF and INH for a further 4 months, either daily or 3 times per week.

Although this treatment regimen has shown 95% cure rate, in areas with a high incidence of MDR-TB, it drops to 50%. In the case of MDR-TB, the WHO recommends the use of DOTS-Plus, which is DOTS plus second-line antitubercular drugs. DOTS-Plus therapy has several disadvantages, such as a treatment duration of 24 months, severe toxicity (due to the second-line drugs) and very high costs (WHO, 2010). Inadequate treatment with second-line drugs may result in extensively drug-resistant tuberculosis.
XDR-TB is developed when mycobacteria become resistant to isoniazid and rifampicin and to certain second-line drugs, in particular to at least one fluoroquinolone and one among kanamycin, amikacin and capreomycin. XDR-TB is not a new phenomenon as it has been recorded previously in Eastern Europe and Central Asia; however, recent outbreaks in at least 45 countries with a high HIV burden have brought this problem to the renewed attention of practitioners and scientists.

Table 1.2: Drugs used for the treatment of TB

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First-line agents</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Rifampicin</strong></td>
<td>Inhibits bacterial RNA synthesis by binding to the β subunit of bacterial DNA-dependent RNA-polymerase leading to blocking of the initiation chain formation in RNA synthesis.</td>
</tr>
<tr>
<td><strong>Isoniazid</strong></td>
<td>A pro-drug activated by katG, exerts its lethal effect through inhibition of synthesis of mycolic acids, through formation of a covalent complex with an acyl carrier protein and beta-ketoacyl carrier protein synthetase.</td>
</tr>
<tr>
<td><strong>Pyrazinamide</strong></td>
<td>Converts to active pyrazanoic acid by pyrazinamidase in susceptible organisms. Pyrazanoic acid inhibits growth of bacterium by lowering pH in the immediate surroundings. Also acts as an antimetabolite of nicotinamide thereby interfering NAD synthesis, inhibiting short-chain, fatty-acid precursors’ synthesis.</td>
</tr>
<tr>
<td><strong>Ethambutol</strong></td>
<td>Inhibits mycobacterial arabinosyl transferases involved in the polymerization of D-arabinofuranose to arabinoglycan, an essential cell wall component</td>
</tr>
<tr>
<td><strong>Aminoglycosides</strong></td>
<td>Irreversible inhibitors of protein synthesis through binding to specific 30S-subunit ribosomal proteins</td>
</tr>
<tr>
<td><strong>Fluoroquinolones</strong></td>
<td>Inhibits bacterial topoisomerase II (DNA gyrase) and topoisomerase IV and thus inhibiting bacterial DNA synthesis</td>
</tr>
</tbody>
</table>
**Bacteriostatic second-line drugs**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-aminosalicylic acid</td>
<td>Anti-metabolite interfering with incorporation of para-aminobenzoic acid into folic acid – folate synthesis antagonist</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>Structural analogue of D-alanine, inhibits incorporation of D-alanine into peptidoglycan pentapeptide</td>
</tr>
</tbody>
</table>

**Other drugs**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clofazimine</td>
<td>Unknown, but may involve DNA binding. Possesses direct antimycobacterial and immunosuppressive properties</td>
</tr>
<tr>
<td>Amoxicillin/Clavulanic acid</td>
<td>Amoxicillin inhibits cell wall synthesis. Clavulanic acid is a beta-lactamase inhibitor</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>Inhibition of protein synthesis via binding to 50S ribosomal RNA as aminoacyl translocation reactions and the formation of initiation complexes are blocked</td>
</tr>
<tr>
<td>Rifabutin</td>
<td>Activity is similar to that of rifampicin. Inhibits bacterial RNA synthesis by binding strongly to the β subunit of bacterial DNA-dependent RNA-polymerase</td>
</tr>
<tr>
<td>Thiacetazone</td>
<td>Not clearly elucidated</td>
</tr>
</tbody>
</table>

TB is treated with a multi-drug regimen and current treatment protocols are lengthy, involving 6 months, thus exceptionally vulnerable to incidences of side effects, unsatisfactory patient compliance and slow improvement of patients. Table 1.3 summarizes the reasons why people die from TB, a curable disease. Therefore, despite the availability of highly effective treatments for TB, cure rates remain low, as commercial anti-TB formulations are inconvenient to administer and patients do not take the prescribed medications with sufficient regularity and duration to achieve a cure. Present efforts in improving treatment focus on shortening the length of treatment or utilizing innovative drug delivery strategies as well as alternative administration routes,
which may play a fundamental role in improving antitubercular chemotherapy efficacy, thereby enhancing patient compliance (Dube et al., 2012b).

Table 1.3: Various factors responsible for the failure of curative treatment of TB

<table>
<thead>
<tr>
<th>Reasons why people die from Tuberculosis : A curable disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>➢ <em>M. tuberculosis</em> finds its victims in developing countries with degraded social and health conditions where access to medicines is limited</td>
</tr>
<tr>
<td>➢ Therapeutic regimen of long duration, patients usually do not take the prescribed medications with sufficient regularity and duration to achieve a cure</td>
</tr>
<tr>
<td>➢ Treatment with a multi-drug regimen. Combined therapies are more effective than single ones, but their fulfillment becomes more difficult for patients and this leads to a poor patient compliance</td>
</tr>
<tr>
<td>➢ Patients have to consume a large number of tablets (up to eight at one time), which is a common cause for non-compliance</td>
</tr>
<tr>
<td>➢ Concomitant presence of conditions compromising the immune system functionality such as HIV infection</td>
</tr>
</tbody>
</table>

1.4. NOVEL DRUG DELIVERY SYSTEMS FOR ANTITUBERCULAR DRUGS

The need for new tools to fight TB, and in particular MDR- and XDR-TB, is pushing towards new strategies and drug candidates to improve therapeutic efficiency. So far, TB therapies have exploited conventional routes of administration with various pharmaceutical forms, such as tablets, capsules and injectable solutions. However, high dosages and frequent administrations are required to maintain the drug therapeutic concentration over time. To try to solve this issue, recent alternatives to conventional oral dosage forms consist of utilizing novel drug delivery systems. Nanotechnology-related rational drug delivery may improve therapeutic success by constraining adverse drug effects and requiring less frequent administration regimes, ultimately resulting in patients who are more compliant and thus, attain higher adherence levels. Further, researchers can
enhance the effectiveness of approved drugs and extend their applicability by providing means to overcome technological limitations such as low bioavailability, resistance, cellular and anatomical barriers, etc. (Dube and Vyas, 2010). Various routes of administration have been explored for delivery of carriers and the advantages and disadvantages of these have been discussed in Fig. 1.5.

Moreover, in spite of the emergence of new antibiotics, treatment of intracellular pathogens such as *Mtb* is difficult since infections are localized within phagocytic cells and most antibiotics, although highly active *in vitro*, do not actively pass through cellular membranes, and hence, it is difficult to achieve the relatively high concentrations of the drugs within the infected cells (Briones *et al.*, 2008; Xu *et al.*, 2006; Liu *et al.*, 2007). The main challenge for intracellular chemotherapy is to design and develop a carrier system for antibiotics that could be efficiently endocytosed by phagocytic cells and, once inside the cells should prolong release of the antibiotics so that the number of doses and associated drug toxicity can be reduced. The associated problems with delivering free antibiotics to the intracellular space have led to the investigations of improved drug carriers for treating intracellular pathogens, including antibiotics loaded into liposomes, microspheres, polymeric carriers, and nanoplexes (Seleem *et al.*, 2009).

Polymeric and lipidic drug delivery systems are well suited as vehicles for the delivery of antimicrobial agents because they usually provide a sustained drug release effect, minimize the toxicity associated with the encapsulated drugs and increase the overall drug efficacy. Moreover, DDS protect the incorporated drug from premature immunological and enzymatic attacks and, in some cases, they act synergistically with cellular bactericidal mechanisms. Another point to be considered regarding the treatment of intracellular infections is the synergy of the polymeric particles with the bactericidal mechanisms of the phagocyte. The stimulation of the intracellular ROI may act
synergistically with the antibiotic activity to kill intracellular bacteria, thus increasing treatment efficiency.

As 80% of TB cases affect the lungs, the site of entrance of the bacilli, inhalable pharmaceutical forms would be extremely useful to obtain high drug concentrations in the lungs and to target directly the alveolar macrophages, the site of residence of the \textit{M. tuberculosis}. As a consequence, the required dose will be remarkably decreased and systemic side effects significantly reduced. Although identifying novel
anti-TB agents remains a priority, the development of novel formulations for currently
cused agents may represent a cost-effective and promising alternative. Achievement of
high local drug concentrations, targeting of alveolar macrophages and the possibility of
prolonging residence time by modifying drug release are the main features which support
the use of the innovative delivery systems.

1.4.1. Drug Delivery Systems

**Liposomes** were discovered in the early 1960s’ by Bangham and co-workers and
subsequently became the most extensively explored drug delivery system. Initially,
though they were used to study *in vitro* simulated biomembrane behaviour, subsequently
they became an essential therapeutic tool most notably in drug delivery and drug
targeting. Structurally, liposomes are phospholipid based colloidal vesicular structures in
which hydrophilic core is entirely enclosed by membranous lipid bilayers. They have the
ability of entrapping both hydrophilic as well as hydrophobic drugs. Up to now, many
studies have been performed to explore their use in targeted and controlled delivery of
various categories of drugs including antimicrobial, antifungal, antiviral and anticancer
agents. Liposomes have also been successfully used for several other practices in drug
delivery such as solubilization of water insoluble drugs, protection of sensitive drug
molecules, alteration of pharmacokinetic and biodistribution and enhancing intracellular
uptake. To increase the targeting potential of liposomes and decrease the RES uptake,
‘stealth liposomes®’ (PEGylated) are the recent innovations in the field of drug delivery.

**Niosomes** are now widely studied as an alternative to liposomes. Non-ionic
surfactant vesicle results from the self-assembly of hydrated surfactant monomers.
Nonionic surfactants of a wide structural variation have been found to be useful
alternatives to phospholipids in the fabrication of vesicular systems. Though the
terminology suggests that distinctions exist between niosomes and liposomes of which
the former is having chemical differences in the monomer units, niosomes possess physical properties, which are similar to liposomes. As the name indicates nonionic surfactant vesicles are prepared by incorporation of components containing nonionic surfactants. However, they may also be prepared with various ionic amphiphiles such as dicetylphosphate, stearylamine, etc., in order to achieve a stable vesicular suspension. The chemical stability as well as the relatively low cost of the materials used to prepare niosomes makes this vesicle more attractive than liposomes for industrial productions both for pharmaceutical and cosmetic applications.

**Nanoparticles** are sub-nanosized colloidal structures composed of synthetic or semi synthetic polymers. The continual quest and maneuvering towards physical stability improvement of liposomes resulted into development of solid core nanoparticles in eighties as an alternative drug carrier. The polymeric nanoparticles can carry drug(s) or proteinaceous substances. These bioactives are entrapped in the polymer matrix as particulates enmesh or solid solution or may be bound to the particle surface by physical adsorption or chemically. The term particulate is suggestively general and doesn't account for morphological and structural organization of the system. Thus they could be nanospheres, nanocapsules, nanocrystals or nanoparticulates. Nanospheres may be defined as solid core spherical particulates, which are nanometric in size. They contain drug embedded within the matrix or adsorbed on to surface; nanocapsules are vesicular system in which drug is essentially encapsulated within the central core surrounded by an embryonic continuous polymeric sheath. In the later, drug(s) is mainly encapsulated in the solution system. The physical chemistry of these systems remains to be the same as of typical colloidal dispersions. The surface charges, dispersibility, density, hydrophobicity and hydrophilicity are some critical factors which ultimately determine the stability characteristics of a system *vis-a-vis* its in vivo disposition.
**Microspheres** are monolithic systems consisting of a polymeric matrix in which the drug substances are either dissolved or dispersed depending on their solubility. However, the terms microcapsules and microspheres are often used synonymously. In addition, some related terms are used as well viz; "microbeads" and "beads". Spheres and spherical particles are also used for microspheres of large size and rigid morphology. The microspheres are characteristically free flowing powders consisting of proteins or synthetic polymers, which may or may not be biodegradable in nature, and ideally having a particle size less than 200 µm. Microspheres represent a very promising drug delivery system to facilitate/achieve target oriented controlled drug release. A number of different substances both biodegradable as well as non-biodegradable have been investigated for the preparation of microspheres. These carriers possess numerous advantages as drug delivery systems.

**Solid lipid nanoparticles** (SLNs) are sub-micron size range colloidal carriers (50-1000 nm), which are composed of physiological lipid, dispersed in water or in an aqueous surfactant solution. SLNs as colloidal drug carrier combine advantages of polymeric nanoparticles, fat emulsions and liposomes simultaneously, avoiding some of their disadvantages. The main features of SLN are the excellent physical stability, protection of incorporated labile drugs from degradation, controlled drug release (fast or sustained) depending on the incorporation model, good tolerability and site-specific targeting. Potential disadvantages such as insufficient loading capacity, drug expulsion after polymorphic transition during storage and relatively high water content of the dispersions (70–99.9%) has been observed.

### 1.1.5. LOCAL DELIVERY TO LUNGS

In recent years, there is renewed interest in formulating drugs for pulmonary delivery for reasons that remain significant. First, the lung mucosa represents a large surface from
which drugs may be systemically absorbed into the bloodstream, without having to
undergo hepatic first-pass. During the process of systemic absorption from the lungs,
drugs introduced into this organ are likely to provide early and high concentrations within
it. This is advantageous if, as in pulmonary TB, the lungs are the intended target site of
drug delivery. Second, lung macrophages are efficient at fulfilling their evolutionary role
of phagocytosing material entering the lungs. It has long been established that
macromolecular drugs and particulate or vesicular drug delivery systems introduced into
the deep lung are likely to be picked up by alveolar macrophages (AM). Finally, it has
been argued that uptake of drug delivery systems by infected macrophages effects rescue
of the macrophage from ‘alternative activation,’ enabling the elaboration of innate
bactericidal responses that could help in killing or containing TB bacilli.

Table 1.4: Summary of work done for antitubercular therapy using novel drug
delivery systems

<table>
<thead>
<tr>
<th>Polymer/Lipid</th>
<th>Drugs</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lecithin</td>
<td>Streptomycin,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amikacin</td>
<td></td>
</tr>
<tr>
<td>ePC</td>
<td>Gentamycin</td>
<td></td>
</tr>
<tr>
<td>PC and phosphatidylglycerol</td>
<td>Sparfloxacin</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>Clofazimine</td>
<td></td>
</tr>
<tr>
<td>ePC, DSPE, PEG</td>
<td>INH, RIF</td>
<td></td>
</tr>
<tr>
<td>DPPC</td>
<td>PZA</td>
<td></td>
</tr>
<tr>
<td>DPPC, DPPG</td>
<td>RFB</td>
<td></td>
</tr>
<tr>
<td>PC, dicetylphosphate</td>
<td>INH, RIF</td>
<td></td>
</tr>
<tr>
<td>DSPC, DPPC, HPC</td>
<td>Capreomycin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O-SAP, Monosialoganglioside</td>
<td></td>
</tr>
<tr>
<td>Complex Type</td>
<td>Components</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>----------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Niosomes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Span 85</td>
<td>RIF</td>
<td></td>
</tr>
<tr>
<td>Span 20, 40, 60, 80, 85</td>
<td>RIF</td>
<td></td>
</tr>
<tr>
<td><strong>Nanoparticles and Microparticles</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLG</td>
<td>INH, RIF</td>
<td></td>
</tr>
<tr>
<td>PLG</td>
<td>RIF, INH, PYZ, ETB</td>
<td></td>
</tr>
<tr>
<td>PBCA, PIBCA</td>
<td>RIF, INH, Streptomyacin</td>
<td></td>
</tr>
<tr>
<td>PIBCA</td>
<td>Ciprofloxacin</td>
<td></td>
</tr>
<tr>
<td>PBCA</td>
<td>Moxifloxacin</td>
<td></td>
</tr>
<tr>
<td>PLGA</td>
<td>RIF, INH, PZA</td>
<td></td>
</tr>
<tr>
<td>Alginate</td>
<td>RIF, INH, PZA, ETB</td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>RIF</td>
<td></td>
</tr>
<tr>
<td>Stearic acid</td>
<td>RIF, INH, PZA</td>
<td></td>
</tr>
<tr>
<td>PLG</td>
<td>RIF, INH, Wheat germ agglutinin, PZA</td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>INH, Mannose</td>
<td></td>
</tr>
<tr>
<td>PLGA, Mannitol</td>
<td>RIF</td>
<td></td>
</tr>
<tr>
<td>PLGA</td>
<td>RIF</td>
<td></td>
</tr>
</tbody>
</table>
PLA & RFB, INH
Hyaluronan & Ofloxacin

**Polymeric Micelles**

<table>
<thead>
<tr>
<th>Micelle Type</th>
<th>Drug</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(CL-GA–PEG-P(CL-GA))</td>
<td>RIF</td>
<td></td>
</tr>
<tr>
<td>MPEG-PLLA and MPEG-PDLA</td>
<td>RIF</td>
<td></td>
</tr>
<tr>
<td>PLA-modified chitosan oligomers</td>
<td>RIF</td>
<td></td>
</tr>
<tr>
<td>Drug-PEG-PAA</td>
<td>INH,</td>
<td>PZA,</td>
</tr>
<tr>
<td></td>
<td>RIF</td>
<td>-</td>
</tr>
</tbody>
</table>

**Dendrimers**

<table>
<thead>
<tr>
<th>Dendrimer</th>
<th>Drug</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPI</td>
<td>RIF</td>
<td>Mannose</td>
</tr>
</tbody>
</table>

1.5.1. Biophysical Basis for Pulmonary Administration

The anatomical organization of the respiratory tract (characterized by extensive bifurcation) and aerosol characteristics of drug molecules (especially particle size) generally determine the reproducibility of pulmonary drug administration.

The respiratory tract comprises the conducting and respiratory regions. The conducting region essentially consists of nasal cavity, nasopharynx, bronchi and bronchioles. Airways distal to the bronchioles and the alveoli constitute the respiratory region, where rapid solute exchange takes place. According to Wiebel’s tracheobronchial classification (Wiebel, 1963), the conducting airways comprise the first 16 generations, and generations 17–23 include the respiratory bronchioles, the alveolar ducts and the alveolar sacs.

The most important parameter that defines the site of deposition of aerosol drugs within the respiratory tract is the particle characteristics of the aerosol. The nature of the aerosol droplets is dependent on its MMAD, which is a function of particle size, shape
and density. Particle charge and air velocities within the airways are also important attributes. Strict control of MMAD of the particles ensures reproducibility of aerosol deposition and retention within desired regions of the respiratory tract. Good distribution throughout the lung requires particles with an aerodynamic diameter between 1 and 5 µm, and thus most inhaled products are formulated with a high proportion of drug in this size range (Chrystyn, 1997). In order to target the alveolar region specifically, the aerosol droplet diameter should not be more than 3 µm. Particles with diameters that are greater than 6 µm are deposited in the oropharynx, whereas smaller particles (<1 µm) are exhaled during normal tidal breathing.

1.6. REVIEW OF LITERATURE

Jain and Vyas, 1995 prepared micro-sized RIF-loaded niosomes containing Span 85 as the surfactant. The authors further extended the investigations and evaluated the biodistribution of niosomes with smaller sizes produced with different sorbitan esters (Span® 20, 40, 60, 80 and 85) and cholesterol (Jain et al., 2006). It was found that the extent of drug entrapped increased gradually with the increase of the hydrophobicity of the surfactant.

Pandey et al., (2003) investigated the pharmacokinetics and antibacterial effect of the nanoparticle bound anti-TB drugs administered via respiratory route in guinea pigs. Nebulization of drug-loaded nanoparticles resulted in plasma-detectable concentrations after 6 h. Biodistribution data revealed that as opposed to the free drugs that were undetectable after 24 h, encapsulated drugs were detected in the lungs until day 11. In nebulization of nanoparticles to M. tuberculosis– infected guinea pigs at every 10th day, no tubercle bacilli could be detected in the lungs after only five doses of treatment, whereas 46 daily doses of orally administered drug were required to obtain an equivalent therapeutic benefit.
Vyas et al., (2004) developed RIF-containing aerosolized micrometric liposomes to target the alveolar macrophages. MBSA and O-SAP were anchored on the surface of the nanocarriers with the intention to improve the selectivity for the lung. Biodistribution showed that all the liposomes, independently of the modification, led to higher concentrations in the lungs and lower concentrations in the plasma when compared to the free drug.

Deol and Khullar (1997) suggested lung-specific stealth liposomes for the targeted delivery of isoniazid and rifampicin. The developed system exhibited controlled release and reduced toxicity in vivo in mice infected with M. tuberculosis.

Dutt and Khuller (2001) investigated PLG microparticles as carriers for INH with sustained drug release and increased bioavailability with single injectable dose. Such depots can show release profiles extending over several months culminating in degradation of the entire polymeric device.

Ain et al. (2002) studied role of PLG in development of a sustained oral delivery system for antitubercular drug(s). Pharmacokinetic parameters were increased when drug were given entrapped in PLG microparticles.

Pandey et al. (2005) studied the chemotherapeutic potential of oral solid lipid nanoparticles (SLNs) incorporating RIF, INH, PZA against experimental tuberculosis. After a single oral administration of nanoparticles to mice, the therapeutic concentrations of all three drugs were maintained in the plasma for 8 days and in the organs (such as lungs, liver and spleen) for 10 days, whereas the free drugs were cleared within 1–2 days.

Pandey and Khuller, 2004 in another study, administered PLG nanoparticles encapsulated with three front-line anti-tubercular drugs subcutaneously to mice. A single subcutaneous dose maintained drug plasma, lungs and spleen concentrations for more than 1 month and led to undetectable bacterial counts in the different organs. The particle
preparation showed a better chemotherapeutic efficacy compared with a daily drug treatment.

**Zhou et al. (2005)** studied microparticle-based lung delivery of INH showing decrease INH metabolism and targeting to alveolar macrophages.

**Zahoor et al. (2005)** studied that when the drug-loaded alginate nanoparticles were administered by nebulization, drug concentrations were detected in the plasma after 3 h, which was faster than PLGA nanoparticles. Following aerosol administration, drug levels above the MIC were detected in the lungs, liver and spleen up to 15 days, compared with just 1 day for the free drugs. Forty-five daily doses of oral free drugs were needed to obtain the same results.

**Saraogi et al. (2010)** prepared RIF loaded gelatine nanoparticle. The nanoparticles not only sustained the plasma level but also enhanced the AUC and mean residence time (MRT) of the drug, suggesting improved pharmacokinetics of drug. Significant reduction in bacterial counts in the lungs and spleen of TB-infected mice was also found.

**Chono et al. (2007)** developed mannose-coated liposomes and found a pronounced increase in the uptake with the mannosylated-nanocarriers as compared to unmodified liposomes. In addition, greater accumulation of modified liposomes was found in the lungs after pulmonary administration to rats.

**Chono et al. (2008)** studied mannosylated Ciprofloxacin-liposomes with 4-aminophenyl-a-d-mannopyranoside (particle size: 1000 nm) and the drug targeting to alveolar macrophages (AMs) following pulmonary administration in rats. Encapsulation of the drug into mannosylated liposomes led to a sharp increase in the uptake by AM from approximately 12% to 22% of dose/mg of cell protein. In addition, biodistribution assays indicated 1.5- and 2.4-fold greater AUC and maximum concentration values, respectively, with the modified liposomes.
Wijagkanalan et al. (2008) studied the *in vitro* and *in vivo* uptake of liposomes (90–125 nm) bearing increasing concentrations of mannose on the surface. It was found that the higher the mannose concentration on the surface, the more pronounced the cellular uptake. Accumulation in alveolar cells after intratracheal administration to rats indicated the higher uptake of mannosylated-nanocarriers, preferably (15–17-fold) by AM over alveolar epithelial type II cells.

O’Hara and Hickey (2000) demonstrated significantly higher efficacy of RIF in PLGA MPs. When compared to free drug, the microparticles led to a significant reduction of spleen inflammation when administered in infected guinea pig. Daily doses of RIF solutions over 10 or 20 days had a positive effect on pulmonary and splenic inflammation but not on the number of viable bacteria in the lungs, while a single administration of particles or 20 days of dosing with free RIF equally decreased the bacteria population in the spleen.

Prior et al. (2005) studied the *in vitro* phagocytosis and monocyte-macrophage interaction with poly-(lactide) and poly-(lactic-co-glycolic acid) microspheres. The results demonstrated that PLA and PLGA microspheres loaded with gentamicin sulfate were efficiently phagocytosed *in vitro*. The end-group uncapped polymer-type microspheres promoted significantly cell activation, which may be of importance for drug delivery and targeting to intracellular infections.

Giovagnoli et al. (2005) incorporated capreomycin in liposomal formulation for pulmonary administration. The vesicles made of distearoylphosphatidylcholine had a narrow size distribution, with a mean diameter lower than 200 nm. They contained 10–13% by weight of the drug, and possessed a smooth surface and spherical/ellipsoidal morphology. These characteristics demonstrated their suitability for use in inhaled formulations.
Ahmed et al. (2008) prepared econazole and moxifloxacin loaded PLG nanoparticles. The prepared systems showed prolonged plasma levels up to 5 and 4 days, respectively. Only 8 doses of nanoparticles individually were sufficient to suppress bacterial clearance in infected mice, in contrast to 56 daily doses of moxifloxacin and 112 doses twice a day of econazole. Furthermore, addition of third drug RIF to this combination showed complete bacterial clearance within 8 weeks.

Garcia-Contreras et al. (2007) prepared large porous particles of Capreomycin for inhalation so as to reduce toxicity.

Chen et al. (2007) synthesized RIF-loaded stereo-complex micelles by the specific assembly of enantiomeric poly (ethylene glycol)–poly(L-lactide) (MPEG-PLLA) and poly(ethylene glycol)-poly(D-lactide) (MPEG-PDLA) block copolymers in a 1:1 ratio of L-PLA- and D-PLA-containing block copolymers. As compared to pure micelles, the RIF loading capacity and encapsulation efficiency of the stereo-complexes were higher.

El-Ridy et al. (2007) carried out biological evaluation of pyrazinamide liposomes.

Hwang et al. (2008) studied the pulmonary delivery of OFX via hyaluronan microspheres for the treatment of tuberculosis. Even though hyaluronan forms a mucoadhesive gel in contact with bronchoealveolar fluids, OFX uptake by macrophages was higher when formulated in this fashion. Intratracheal administration of OFX-loaded hyaluronan particles resulted in 50% lower serum bioavailability with respect to intravenous or oral OFX.

Verma et al. (2008) investigated intracellular concentrations of INH and rifabutin resulting from administration of inhalable microparticles of these drugs to phorbol-differentiated THP-1 cells and the pharmacokinetics and biodistribution of these drugs upon inhalation of microparticles or intravenous administration of free drugs to mice.
Ohashi et al. (2009) produced RIF loaded biodegradable PLGA that were incorporated into mannitol microspheres. The composite particles possess the advantages of nanoparticles without their drawbacks (e.g., poor aerosolization). Encapsulation of the nanoparticles in mannitol improved the in vivo uptake of the drug by alveolar macrophages in rat lungs as compared to RIF-containing PLGA and mannitol microspheres.

Sharma et al. (2004) found that administration to infected guinea pigs of nebulized RMP, INH, and PZA coencapsulated in wheat germ agglutinin-functionalized PLG nanoparticles was even more effective.

Sharma et al. (2007) investigated whether inhalable microparticles containing two anti-tuberculosis agents, INH an RIF, evoke host-defence strategies in macrophages in addition to targeting the incorporated drugs.

Brandhonneur et al. (2009) evaluated the influence of ligand grafting on the rate and intensity of uptake of PLGA microparticles by alveolar macrophages. This work showed that the uptake of negatively charged ligand-grafted microspheres (−26 to −51mV) was increased up to two to four times according to the ligand compared to ungrafted microspheres (−81mV) and displayed saturation as opposed to the cationic PLL-grafted microspheres. Furthermore, this work clearly showed that the relative contribution of specific and non-specific processes to the overall uptake varied greatly according to the ligands, and was dependent on the particle-to-cell ratio.

Hirota et al. (2010) encapsulated RIF in PLGA microspheres and administered to TB-infected rats. The study showed that particles were taken up very efficiently by macrophages inducing a potent bactericidal effect. Phagocytosis of RIF loaded PLGA MS does not generate the toxic humoral factors to AMs, such as TNF-α and NO, and the
phagocytosis does not affect the viability of AMs, showing that, PLGA MS are not toxic to AMs.

**Onoshita et al. (2010)** developed a pulmonary drug delivery system for the treatment of tuberculosis using RIF encapsulated in PLGA microspheres the behavior of RFP-PLGAMS. It was shown by fluorescent microscopic studies that the RIF-PLGA MS taken up by the NR8383 cells were localized in phago-lysosomes and then degraded. It was considered, therefore, that RFP was released into the cytosol with drug potency intact.

**Saraogi et al. (2011)** prepared mannosylated gelatin nanoparticles for the selective delivery of INH to alveolar macrophages and concluded that gelatin nanoparticles can be explored as a potential carrier for safer and efficient management of TB through targeted delivery.

### 1.7. RESEARCH ENVISAGED

TB has been a major infectious disease throughout human history and changed its grasp as human kind advanced industrially and technologically. TB has become a significant opportunistic disease among populations with a high incidence of AIDS. TB is most often due to *Mtb*, and the lungs are the primary site of infection for the systemic pathogen but it can also affect central nervous system (meningitis), lymphatic system, circulatory system (Miliary TB), genitourinary system, bones and joints. Among the various forms of TB, pulmonary TB is most commonly characterized by the involvement of alveolar macrophages harboring a large number of tubercle bacilli. Even today, more than one hundred year after its first description, TB is still a great health problem worldwide. Globally, there were an estimated 8.8 million incident (new) cases of TB in 2010 including 1.1 million cases among people with HIV. In 2010, 1.4 million people died from TB, including 350,000 people with HIV, equaling 3,800 deaths a day. Although
potentially curative treatments are available for almost half a century, still TB remains the leading cause of preventable deaths.

RIF and INH are used as a first line drugs for the treatment of TB. Both the drugs when used in combination lead to eradication of bacilli. The mechanism of action of these drugs is different, making these drugs a preferred combination for a successful therapy. But multi-drug regimen and current treatment protocols are lengthy, involving 6 months, thus exceptionally vulnerable to incidences of side effects, unsatisfactory patient compliance and slow improvement of patients. Therefore, despite the availability of highly effective treatments for TB, cure rates remain low, as commercial anti-TB formulations are inconvenient to administer and patients do not take the prescribed medications with sufficient regularity and duration to achieve a cure. Present efforts in improving treatment focus on shortening the length of treatment or utilizing innovative drug delivery strategies as well as alternative administration routes, which may play a fundamental role in improving anti-TB chemotherapy efficacy, thereby enhancing patient compliance.

In the development of anti-TB therapy, two points are important; first, the rate of metabolism of \textit{Mtb} is slow, resulting in generation time that is measured in hours. Therefore, drug regimes should ideally have a low level of toxicity for long term administrations. Secondly, the bacillus is a facultative intracellular parasite; therefore drug should also be able to penetrate host cells. Thus, an ideal targeted drug delivery system would be one that is not only to deliver drugs for long term but also be able to target the drugs to the intracellular environment of host cells. Apart from these two important points we can also alter the activation status of the macrophages, cells where \textit{Mtb} resides.
TB management may be improved with the introduction of longer acting formulations releasing the antimicrobial agents in a slow and sustained manner, which would allow reduction in frequency and dosing numbers. During the last three decades, therapeutic systems based on polymers, both natural and synthetic, have shown to be effective in controlling rate or time of drug release, in enhancing drug targeting specificity while lowering systemic drug toxicity and providing protection for pharmaceuticals against degradation. Synthetic biodegradable polymers have gained more popularity than natural biodegradable polymers. The major advantages of synthetic polymers include high purity of the product, more predictable lot-to-lot uniformity, and free of concerns of immunogenicity.

During the last 30 years, numerous biodegradable polymers have been synthesized. Most of these polymers contain labile linkages in their backbone such as esters, orthoesters, anhydrides, carbonates, amides, urethanes, etc. Among the different classes of biodegradable polymers, the thermoplastic aliphatic poly(esters) such as poly(lactide) (PLA) and its glycolic acid copolymer poly(lactide-co-glycolide) (PLGA) are most commonly used as drug carrier due to their excellent biocompatibility and biodegradability and mechanical strength. They can degrade by non-enzymatic hydrolysis of the ester backbone in body fluid. The degradation products (i.e. lactic and glycolic acids) are metabolic compounds. Most importantly, PLA and PLGA have been approved by the United States Food and Drug Administration (FDA) for drug delivery. Because of its biodegradability and biocompatibility, poly (lactide-co-glycolide) (PLGA; a synthetic polymer) has been a popular choice as a drug carrier. Designing formulations using these polymers can fulfill our first objective of sustaining the drug action of anti-TB drugs.

Most of the anti-TB drugs presently in use, fail to penetrate macrophages within which bacilli lurk; utilizing delivery systems and their engineered versions can be
therapeutically effective approach. Macrophages offers the opportunity for developing surface decorated delivery systems as various receptors such as carbohydrates, lectins, peptides, glycoproteins, glycolipids, scavenger, etc. Polymeric nanoparticles can be actively targeted via easier ligation with site-specific ligands that in turn enhance target specificity and performance efficiency. Targeted drug delivery systems can optimize the therapeutic index of anti-TB drugs by increasing the drug concentration ratio of diseased tissue to normal tissue.

Apart from this, there is renewed interest in formulating drugs for pulmonary delivery. First, the lung mucosa represents a large surface from which drugs may be systemically absorbed into the bloodstream, without having to undergo hepatic first-pass. During the process of systemic absorption from the lungs, drugs introduced into this organ are likely to provide early and high concentrations within it. This is advantageous if, as in pulmonary TB, the lungs are the intended target site of drug delivery. Phagocytosed nanoparticles potentially can deliver larger amounts of drug to the cytosol than oral doses. Second, lung macrophages are efficient in fulfilling their evolutionary role of phagocytosing material entering the lungs. It has long been established that macromolecular drugs and particulate or vesicular drug delivery systems introduced into the deep lung are likely to be picked up by alveolar macrophages.

Particle endocytosis is sufficient to induce a ‘classical’ activation response in the macrophages, which mobilizes intracellular and extracellular calcium, undergoes a respiratory burst, generates free radicals and initiates mechanisms to acidify the phagosome, induces phagosome-lysosome fusion or autophagy, and thereby destroys and digests the foreign particle. Various surface receptors present on macrophages when stimulated induce high levels of proinflammatory mediators like Th1 cytokines, generation of reactive nitrogen intermediate (RNI) and reactive oxygen species (ROS).
Classically activated macrophages with high levels of inflammatory mediators possess improved capacity of killing intracellular bacteria, such as *Mtb*. Finally, it has been argued that uptake of drug delivery systems by infected macrophages effects rescue of the macrophage from ‘alternative activation,’ enabling the elaboration of innate bactericidal responses that could help in killing TB bacilli.

The intention of this work is to adduce scientific rationale for incorporating into the standard treatment of TB, the objective of activating lung macrophages (e.g. by inhalable particles containing stimulators), so that host-defense responses may also be co-opted to combat infection.
1.8. PLAN OF WORK

The proposed work was planned to be carried out as follows:

1. Exhaustive literature survey

2. Selection of drug(s), polymer and ligand

3. Preformulation and analytical methods
   - Identification of drug(s)- Physical identification, UV and IR spectroscopy
   - Drug estimation in various solvents and biological fluids
• Drug-excipient interactions

4. Preparation and characterization

• Preparation of colloidal carriers using various formulation and process variables employing drug(s)

• Preparation of ligand conjugated colloidal carriers

5. In vitro characterization of plain and ligand conjugated nanoparticles

• Size and size distribution

• Shape and surface morphology

• Surface charge

• Drug entrapment efficiency

• Presence of ligand on the surface

• In vitro drug release

6. Stability studies

7. Ex vivo studies

• Cell toxicity assay

• Cell uptake study

• Antitubercular activity

• Estimation of cellular markers

8. In vivo studies

• Biodistribution study

• Blood/plasma drug concentration study

• Antitubercular activity

• Hepatotoxicity study

9. Statistical analysis of data, interpretation of results, summary and conclusion
Fig. 1.3: Stages of granuloma formation in TB. The initial stage of TB is characterized by expansion of the bacterial population in the absence of adaptive immunity. When initiation of adaptive immunity eventually occurs, CD4+ and CD8+ effector T lymphocytes are recruited to infected tissue and curtail bacterial growth. The mature granuloma represents equilibrium between virulent mycobacteria and the host immune response.
Fig. 1.4: *Mtb*-Macrophage interaction. The mycobacterial phagosome is formed upon entry of mycobacteria and remains accessible to the extracellular environment, e.g., for the acquisition of iron through the transferrin receptor (TfR). Once inside phagosomes *Mtb* secretes the phosphatase SapM and the serine/threonine kinase PknG to prevent phagosome-lysosome fusion. The mycobacterial phagosome (light-yellow circle) is also prevented from fusing with lysosomes by the active recruitment of the host protein coronin 1/TACO (purple). Lysosomal-associated membrane protein 1 (LAMP1) and vacuolar type H⁺-ATPase (V-ATPase) are components of the lysosome and prevents fusion with the endosomal pathway. The arrest of phagosomal maturation is, however, incomplete and some phagosomes mature to form phagolysosomes. Phagosome maturation is promoted in activated macrophages, particularly after IFN-γ stimulation.