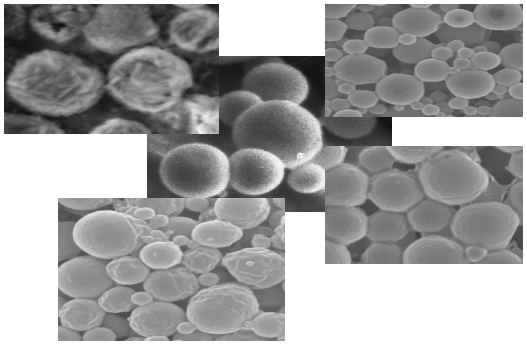
European Union *Ministero dell'Università e Ricerca* *University of Cagliari*

UNIVERSITY OF CAGLIARI

DEPARTMENT FARMACO CHIMICO TECNOLOGICO



CHITOSAN AND PLGA MICROSPHERES AS DRUG DELIVERY SYSTEM AGAINST PULMONARY MYCOBACTERIA INFECTIONS

PhD Program Coordinator:

Prof. Gianni Podda

Supervisors:

Prof. Anna Maria Fadda

Dr. Donatella Valenti

PhD Candidate:

Maria Letizia Manca

PhD Program in:

Tecnologie e legislazione del farmaco e delle molecole bioattive XVIII ciclo

S.S.D Chim/09

Acknowledgments

First of all I woukd like to thank my Professor Anna Maria Fadda for excellent scientific guidance, fruitful discussion, providing me with lab space and equipment and sending me to a number of conferences.

I want to express my sincere gratitude to Dr. Donatella Valenti for all the support and guidance throughout my time as PhD student. I would also like to thank her for her enormous generosity when I need it.

I would like to express my gratitude to all collegues at the Department Farmaco Chimico Tecnologico, in particular Chiara, Maria, Francesco, Carla, Simona, Francesca and Salvatore, for all unconditioned help given during my time in the institute

Riccardo Scateni, many thanks for helping in solving computer problems.

My thanks go to Professor Sophia Antimisiaris for giving me the opportunity to spend one fruitfull year in her laboratory in Greece. In particular I would like to thank all collegues at the University of Patras, Voula, Catalina and George for always having time for creating an enjoyable and stimulating atmosphere.

Last but not least, no word can express my sincere gratefulness to my family. Thank you all for your support during the time of this thesis work.

European Union, Social Funds, PON 2000-2006, and Marie Curie Early Stage Scholarship Program (Project name: Towards a Euro-PhD in advances drug delivery, Contract No: MEST-CT-2004 – 504992) are gratefully acknoweledged for the financial support and the personal fellowship.

Table Of Contents

1. General Introduction

1.1. Pulmonary Mycobacterial Infections and Immuno-Compromised Hosts

Pulmonary infections, due to a variety of pathogens, are important causes of morbidity and mortality today especially in immuno-compromised individuals.

The main consequence of HIV infection is a progressive depletion and disfunction of T cells, with defects in macrophage and monocyte functions that have a central role in anti-mycobacterial defences. Susceptibility to opportunistic pathogens has also been found in patients with congenital T cell defects and in people treated with chemotherapeutic agents or irradiation that cause T cell depletion. Most prominent among these infections is pneumonia due to Pneumocystis Carinii but there has also been a high incidence of mycobacterial infections (1,2).

Mycobacterial infection has been documented as a common opportunistic disease in patients with acquired immunodeficiency syndrome (AIDS). At Memorial Sloan-Kettering Cancer Center more than 400 patients with AIDS have been followed. There have been 90 documented cases of disseminated Mycobacterium Avium Complex (MAC) infection, 11 cases of disease due to Mycobacterium Tuberculosis (MTB), and one case of combined MAC-MTB infections (3).

Tuberculosis (TB) has become a significant opportunistic disease among populations with a high incidence of AIDS. TB is most often due to Mycobacterium tuberculosis (MTB), and the lungs are the primary site of infection for the systemic pathogen but MTB can also affect central nervous system (meningitis), lymphatic system, circulatory system (Miliary tuberculosis), genitourinary system, bones and joints, (4). Among the various forms of tuberculosis, pulmonary tuberculosis is most commonly characterized by the involvement of alveolar macrophages harboring a large number of tubercle bacilli. The bacilli secrete molecules that prevent phagosome–lysosome fusion. Moreover, due to their very hydrophobic waxy cell wall, bacilli are resistant to digestion by lysosomal enzymes and hence resist the killing effects of macrophages. Inside the macrophages the bacteria will be either destroyed or begin replicating, or remain latent indefinitely. If replication is not prevented, the bacilli multiply and may eventually cause the macrophage to break. Problems created by bacterial infection are linked to their ability to survive and multiply inside the body, especially in the lungs, and to the natural immune response of the infected host.

Even today, more than one hundred year after its first description, TB is still a great health problem worldwide. It is estimated that there are 1 billion persons infected with tuberculosis worldwide, with 8 million new cases and 3 million deaths per year (5). Such high mortality rate seems to be due to progressive HIV infection rather than TB, and the degree of immunosuppression being the most important predictor of survival of HIV-TB patients. MTB probably increases HIV replication by inducing macrophages to produce transactivating cytokines (TNFα, IL-1, IL-6).

However, immunocompromised patients are also at risk of mycobacteria other than tuberculosis (MOTT) infections. Recent reports have described several groups of patients with acute leukemia, lymphoma, visceral malignancies and treated with immunosuppressive therapy who have been infected with MOTT such as M. avium, M. fortuitum, M. chelonae, M. scrofulaceum and M. hemophilum (6, 7). Mycobacterium Avium Complex (MAC) can cause pulmonary disease, subacute lymphadenitis and disseminated diseases (8). MAC was also recognised in the AIDS pandemic as a cause of serious disseminated infection and was the most common cause of systemic bacterial infection in AIDS, affecting more than 50% of patients in the developed countries (9). In AIDS patients with disseminated infection, the mononuclear phagocyte system is the predominant site of infection, but other organ systems such as skin, bone and joints, eyes, thyroid, adrenals, testis and the central nervous system can be infected (10, 11). Bacteremia occurs in most of these patients, the organism being predominantly in circulating monocytes. Monocytes and fixed tissue macrophages are full of MAC in AIDS patients, indication of the immune deficiency in these individuals (11, 12, 13, 14, 15). Furthermore, epidemiological studies suggest that MAC strains associated with pulmonary disease may differ from those associated with disseminated disease in AIDS patients. Although if the prevalence of disseminated MAC associated disease was high, great individual susceptibility, and geographic and seasonal variations has been described. For instance, disseminated MAC is rare in Africa, although MAC is prevalent in soil and water samples from the area where advanced AIDS patients are present (16). This phenomena remains unexplained, although it is postulated that widespread previous antimycobacterial immunity from the high rate exposure of Africans to MTB and BCG vaccination may be responsible. This is supported by data that suggest that prior TB diagnosis may be somehow protective against disseminated MAC (17). Pathogenesis of MAC infection is incompletely understood. Disseminated MAC is usually believed to follow primary acquisition of the mycobacteria. It appears that MAC first colonizes the gastrointestinal (GI) tract or respiratory

mucosa, and then dissemination follows. In contrast to the immuno-compromised host, in which MAC disease is usually limited to the lungs, in patients with AIDS, bacteremia is by far the most common syndrome. Before the 1980s, infections with these bacteria were uncommon in humans and were recognized as a slowly progressing pneumonitis in elderly patients with chronic pulmonary disorders, particularly in patients with silicosis (18) and, occasionally, immunocompromised leukemic patients (19). Since 1981, however, numerous medical institutions began to report cases of MAC bacteremia in AIDS patients (20) which were responsible for significant morbidity and mortality in human immunodeficiency virus (HIV)-positive patients (21). Disseminated infections with MAC organisms are diagnosed in only 3% of HIV-positive patients at the time of AIDS diagnosis (22). However, these infections are found in about half of the autopsied patients with AIDS (23). MAC isolates are intracellular pathogens resistant to many of the standard antituberculosis drugs. In many cases this resistance is due to the low levels of drug permeation into macrophages, as many antibiotics are unable to traverse the cell membranes, making it difficult to achieve sufficient concentrations at the infection sites (24, 25).

Nevertheless, it was not until the post-human immunodeficency virus (HIV) era, that renewed interest became widespread in mycobacterial diseases in the immunocompromised host. Several reasons for such interest are the following. First, re-emergence of TB in countries where this type of disease was in the way of eradication, was puzzling. Second, MOTT that were generally quite rarely isolated before the advent of AIDS, but represented before the recently used efficient antiretroviral therapies, have a considerable role in morbidity and mortality. This was particularly true for the M. avium complex (MAC) because these organisms are the single most important cause of disseminated bacterial infection in AIDS patients (26). Third the number of cases of infection with multiply-drug-resistant (MDR) of MT and MAC is increasing and this has compromised both treatment and control programmes worldwide. The rising prevalence of MDR strains has resulted in outbreaks and individual cases that are a problem to treat and are often fatal. Worldwide, TB and disseminated MAC disease, both contribute substantially to morbidity and mortality in this population.

Among the mycobacterial genus, the vast majority of species are saprophytic belonging to the environmental microflora. They are present at different latitudes, and could be isolated from soil, and water. Mycobacteria are aerobic, Gram-positive, non-motile bacilli with a high mycolic acid content in their cell wall that enables intracellular survival within mononuclear phagocytes.

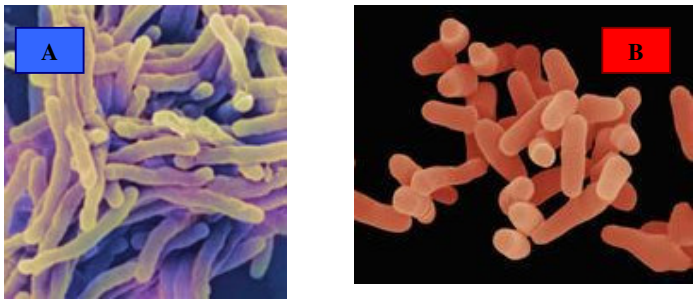


Figure 1.1:

Mycobacterium Tuberculosis MT (A) and Mycobacterium Avium Complex MAC (B)

Being intracellular survivors, Mycobacteria species evoke a granulomatous to pyogranulomatous host response. Mycobacteria can be grouped conceptually into three categories: (1) obligate parasites that behave as primary pathogens and require a mammalian host to that comprise the tubercle bacilli (Mycobacterium tuberculosis (MTB), Mycobacterium bovis (MB) and Mycobacterium microti(MM)), (27, 28, 29, 30, 31); (2) saprophytes that can behave as facultative pathogens, causing localised or systemic disease depending on the degree of host compromise; these can be divided further into slow growers, such as the Mycobacterium avium intracellulare complex (MAC), Mycobacterium genavense (MG) and Mycobacterium xenopi (MX), or rapid growers, such as Mycobacterium fortuitum (MF), Mycobacterium chelonae (MC), Mycobacterium smegmatis (MS), Mycobacterium phlei (MP) and Mycobacterium thermoresistible (MTH); and (3) Mycobacteria species so difficult to culture that their environmental niche has not been determined with certainty, including Mycobacterium leprae (ML), Mycobacterium visibilis (MV),(31, 32, 33, 34, 35).

The natural history of pathological and casual opportunist mycobacteria diseases differ due to different tissue tropisms: the MOTT opportunists appear to be more limited than M. tuberculosis in parallel with the experimental observations of more limited virulence. For instance, experimental infection with M. tuberculosis is often lethal in normal non-immuno-compromised mice, in contrast virulent strains of M. avium infection are only lethal in immunodeficient mice (36).

MTB was first described on 1882 by Robert Koch, and the M. Tuberculosis genome was sequenced (37, 38) in 1999. MTB divides every 15 to 20 hours, extremely slowly compared to other bacteria, which tend to have division times measured in minutes. It is a small, rod-like bacillus that can withstand weak disinfectants and can survive in a dry state for weeks but can grow only within a host organism. An important consideration in the treatment of tuberculosis

is the fact that the etiological agent, MTB, has the ability to persist intracellularly in the host macrophage for long periods of time. This becomes even more important when one considers the ability of MTB to persist in a dormant state, thus giving rise to a large group of infected individuals who carry the organism in a subclinical state without having active disease.

The term MAC was coined after debate as to whether Mycobacterium intracellulare represented a distinct species or was merely a variant of M avium (39, 40, 41). Traditionally, the MAC has been divided into serotypes using agglutination reactions, immunodiffusion, skin testing with sensitins, biochemical reactions on culture, or a combination of these (41). Molecular techniques have more recently been used to clarify the MAC group (42, 43). M. avium and related species are ubiquitous, saprophytic organisms commonly found in surface waters such as salt or fresh-water marshes, ponds, lakes, or soil. Animals, including people, are commonly exposed to these organisms (41). M. avium may give rise to disease if introduced in sufficient numbers through a breach in the skin or via alveolar deposition. In immune competent hosts, such events would cause localised infections, although generalised disease may arise in patients with compromised cell-mediated immunity (13).

Even though the availability of powerful anti-tubercular drugs (ATD) such as rifampicin (RFP), isoniazid (INH) and pyrazinamide (PZA) makes TB and MOTT infections, curable diseases, the latter is far from eradication, the main reason being that multiple anti-tunercular dugs (ATD) need to be administered for 6-9 months. Patients often find it troublesome to begin their day with a mouthful of pills. Further, as clinical symptoms improve, they may not consider the need to continue ATD and may actually forget to take the drugs. All these factors result in non-compliance and eventually lead to therapeutic failure.

1.2. Alveolar Macrophages and MTB/MAC

MTB and MAC are facultative intracellular micro-organisms that can survive and multiply intra-cellularly and are protected from host defence mechanism. They interact mainly with macrophages in the alveolar space of the lung, where they are able to invade and replicate in both cell types. Their intracellular location serves as reservoir which is thought to be of importance in recurrent infections.

Macrophages are present in all major compartment of the body and they are particularly involved in removing foreign particles and micro-organism from the blood. After being removed from the blood, the behaviour of the micro-organism in the macrophage becomes of great importance. Killing of the micro-organism could mean termination of the infection,

whereas microbial persistence and growth in the macrophage could lead to infection in the organ harbouring the macrophages, and hence to current infection of the blood.

Macrophages are found at all epithelial lung surfaces but are far more frequent in the deeper regions of the lung (44). The number of macrophages in the lung is variable and can be raised on exposure to certain materials, for example cigarette smoke (45, 46, 47). In the normal human lung, however, macrophages comprise of over 95% of the mobile cell population and account for 2–5% of the total alveolar cells numbering between 50 and 100 per alveolus (45, 48).

Macrophages are normal motile residents of the airways, interstitial matrix, and alveolar regions of the lungs (49). Particles deposited in the alveolar region are taken up rapidly by macrophages. Phagocytic times of a few minutes (50) up to an hour (51) have been reported. The contribution of pulmonary endocytosis to the overall lung clearance is determined by the particle size and particle shape, (52) solubility, particle burden, (53, 54) and the chemical nature of the inhaled aerosol. Alveolar macrophage-mediated clearance is a much slower process than mucociliary clearance, with retention half-times in the range of 50–80 days in rats and about 10 times longer in humans (55). Particle phagocytosis by alveolar macrophages can be: 1) fast and efficient (titanium dioxide, diameter < 0.2 mm), 2) not efficient (ultrafine particles), 3) incomplete (long fibers cannot be completely phagocytized by a spherical cell with a diameter of approximately 12 mm), or 4) overloaded (ie, when particles occupy a large fraction of the volume of individual alveolar macrophages) (56). Alveolar macrophages can clear particles from the alveolar region in 4 ways: 1) transport along the alveolar surface to the mucociliary escalator, 2) internal enzymatic degradation, 3) translocation to the tracheo-bronchial lymph, and/or 4) combination of the interstitial lymphatic route and mucociliary transport. It is believed that translocation of particle-laden macrophages to the mucociliary region is responsible for the initial rapid clearance of insoluble particles in the first 24 hours after deposition (57). The enzymatic activity following phagocytosis by alveolar macrophages is well known (58, 59) and its contribution to the overall pulmonary clearance requires consideration for enzyme-sensitive compounds such as biomolecules. Lung surfactant may cause large molecules to aggregate, which could enhance ingestion and digestion by alveolar macrophages (60).

1.3. Lung Anatomy

The lung is the body's organ of respiration. The respiratory tract can be divided into upper and lower airways, with the line of division being the junction of the larynx and trachea (61).

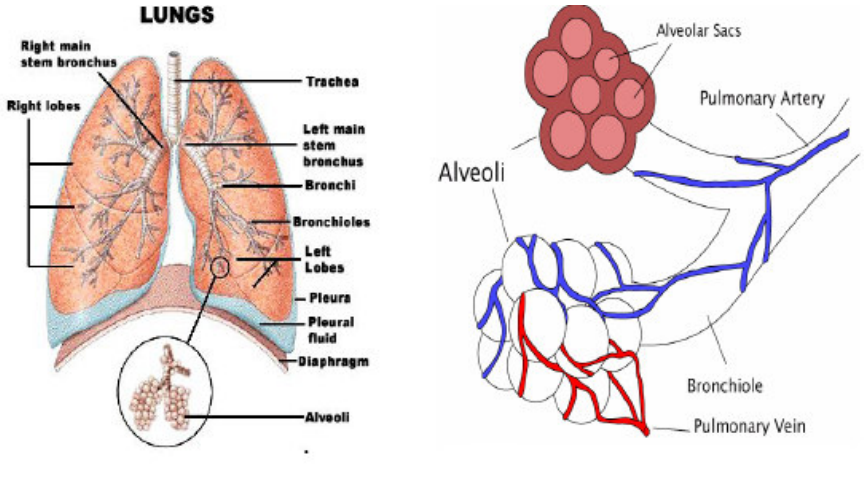


Figure 1.2: The Anatomy Of Human Lungs

The upper airways or nasopharyngeal region consists of the nose, mouth, larynx, and pharynx. Below the contours of the nasopharyngeal region, the lower airways resemble a series of tubes undergoing regular dichotomous branching (62). Successive branching from the trachea to the alveoli reduces the diameter of the tubes, but markedly increases the surface area of the airways, which allows gas exchange (63, 64). The lower airways can be divided into 3 physiologic zones: conducting, transitional, and respiratory zones (62, 65). The conducting zone consists of the larger tubes responsible for the bulk movement of air and blood. In the central airways, air flow is rapid and turbulent and no gas exchange occurs. The transitional zone plays a limited role in gas exchange. The epithelial layer of the trachea and main bronchi is made up of several cell types, including ciliated, basal, and goblet. On the surface of the epithelium of the proximal respiratory tract, ciliated cells predominate. A large number of mucus-producing and serum-producing glands are located in the submucosa.

The human lung consists of 5 lobules and 10 bronchopulmonary segments. Arranged adjacent to each segment are lung lobules composed of 3–5 terminal bronchioles. Each bronchiole supplies the smallest structural unit of the lung, the acinus, which consists of alveolar ducts, alveolar sacs, and alveoli. Alveolar epithelial type I cells represent the principle cell type lining the surface of the alveoli. The major functions of these cells, which cover 93% of the

alveolar space, are to provide a surface for gas exchange and to serve as a permeability barrier. Alveolar epithelial type II cells have a much smaller surface area per cell and they represent 16% of the total cells in the lung. They play a basic role in synthesis, secretion and recycling of surface-active material (lung surfactant).

The alveolar blood barrier in its simplest form consists of a single epithelial cell, a basement membrane, and a single endothelial cell. While this morphologic arrangement readily facilitates the exchange, it can still represent a major barrier to large molecules. Before entering the systemic circulation, solutes must traverse a thin layer of fluid, the epithelial lining fluid. This layer tends to collect at the corners of the alveoli and is covered by an attenuated layer of surfactant.

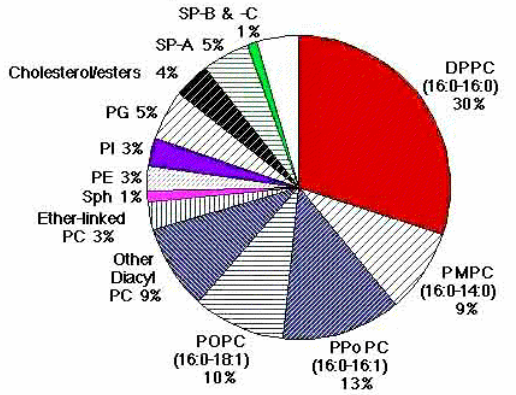


Figure 1.3: Lung Surfactant Composition

Unlike the larger airways, the alveolar region is lined with a surface active layer consisting of phospholipids (mainly phosphatidylcholine and phosphatidylglycerol) (66) and several key apoproteins (67). The surfactant lining fluid plays an important role in maintaining alveolar fluid homeostasis and permeability, and participates in various defence mechanisms. Recent studies suggest that the surfactant may slow down diffusion out of the alveoli (68, 69). The respiratory airways, from the upper airways to the terminal bronchioles, are lined with a viscoelastic, gel-like mucus layer 0.5–5.0 mm thick (70). The secretion lining consists of two layers: a fluid layer of low viscosity, which surrounds the cilia (periciliary fluid layer), and a more viscous layer on top, the mucus (71). The mucus is a protective layer that consists of a complex mixture of glycoproteins released primarily by the goblet cells and local glands (72). The mucus blanket removes inhaled particles from the airways by entrapment and mucociliary transport at a rate that depends on viscosity and elasticity (73). The lung tissue is

highly vascularized, which makes pulmonary targeting difficult because of fast absorption of most drugs (especially lipophilic and low molecular weight drugs).

1.4. Pulmonary Drug Delivery Following Aerosol Therapy

There are several advantages in delivering drugs, such as antimicrobial agents, to the lungs including a non invasive method of delivery; a large surface area for absorption (~75m^2); thin (0.1 to 0.5 μm) alveolar epithelium, permitting rapid absorption; absence of first-pass metabolism; rapid onset of action; and high bioavailability.

Since the advent of nebulizer therapy in 1859, nebulizers have been used to treat a range of pulmonary diseases in pediatric and adult populations, including asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF).The expansion of nebulizer therapy in the mid to late 20th century for common respiratory diseases has been followed by a focus on use for more specific indications and certain new applications.

The development of an inhalant therapy that is efficacious and safe depends not only on a pharmacologically active molecule, but also on a well-designed delivery system and formulation. It is the optimization of the whole system (drug, drug formulation and device) that is necessary for the successful development of inhalation therapies, both new and old, for the treatment of local and systemic diseases. Drug–device combinations must aerosolize the drug in the appropriate particle size distribution and concentration to ensure optimal deposition and dose in the desired region of the lung.

Although the traditional form of inhalation therapy dates back to the earliest records of ancient cultures, the advantages of inhalation therapy have essentially remained the same. Several studies (1–3) have demonstrated the clinical advantage of inhalation aerosols over systemic therapy for the treatment of lung disorders. Relatively small doses are required for effective therapy, reducing systemic exposure to drug and thus minimizing adverse effects. Delivering small doses of active ingredients directly to the lung effectively targets the drug, thereby maximizing therapeutic effect while minimizing adverse effects.

1.4.1. Delivery Device: Jet Nebulizer

Nebulizers have been used for many years to treat asthma and other respiratory diseases. There are two basic types of nebulizer, jet and ultrasonic nebulizers. Ultrasonic nebulizers utilize high frequencies to convert liquid into a fine mist (74).

The jet nebulizer functions by the Bernoulli principle by which compressed gas (air or oxygen) passes through a narrow orifice creating an area of low pressure at the outlet of the adjacent liquid feed tube. This results in drug solution being drawn up from the fluid reservoir and shattered into droplets in the gas stream. Jet nebulizers produce smaller droplets than do pMDIs, and these smaller droplets penetrate more easily to the small airways (74).

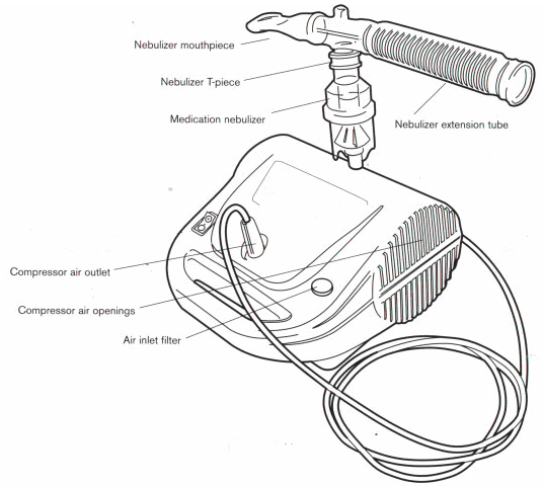


Figure 1.4: Jet-Nenulizer Device

The choice of a proper nebulizer system is very crucial for the efficient delivery of aerosolized respiratory drugs (75). Jet nebulizers are widely used, because of their durability, ease of maintenance, and availability at low cost. In a jet nebulizer, compressed air is forced through a narrow orifice, which leads to a decrease in lateral pressure, thereby drawing up liquid from the feed tube. The primary droplets are produced in the nozzle region and then broken down into smaller droplets. The non-respirable droplets are removed by baffles (76). The droplets may increase in diameter because of condensation of water vapor. This condensation occurs because the droplets are at a low temperature (about 10°C) after being released from the nebulizer, if the surrounding air stream was unsaturated (77).

Fewer than 0.5% of the resultant droplets are small enough to leave the nebulizer; the larger droplets drip back into the liquid to start the process again. Size distribution data for jet nebulizers come from a number of studies (78, 79, 80, 81, 82, 83). These kind of nebulizer are able to produce, from physiologically isotonic saline, initial droplets with aerodinamic diameter from 1.6 to 4.9 μm.

The size distribution of aerosol droplets from a jet nebulizer depends on the diameter of the liquid inlet orifice, the air velocity at the nozzle, and the ratio of the mass flow rate of air to

liquid (84). The droplets at the nebulizer outlet become smaller with increasing air velocity and increasing ratio of the mass flow rate of air to liquid. Increasing the nebulizer flow rate increases the aerosol output rate (78). The formation of droplets depends on the proper alignment and placement of the jet nozzle, liquid inlet, and impaction surface; many nebulizers have baffles to remove larger particles by impaction. Thus, the internal orientation and geometry of the jet nebulizer is critical to maintaining a reproducible output and size distribution. The output and size distribution are also affected by droplets' impacting and settling onto the tubing downstream of the nebulizer (79). Other factors that in some situations affect the droplet size are the gas density and the liquid surface tension and viscosity (84). The solution concentration itself has no effect on the size distribution of the droplets except to the extent that it changes the physical characteristics of the solution (78, 80). The nebulizing action causes continual reflux of a large volume (and surface area) of water, which promotes extensive water evaporation. Evaporative losses from the solution can be decreased by using air from a compressor, which supplies air at ambient humidity, rather than from a compressed gas cylinder, which supplies air that is totally dry. In summary, the size and solute concentration of droplets are affected by any difference in vapor pressure between the droplet and the surrounding air and the time available for equilibration. Thus gradients of relative humidity and temperature do affect the droplets and can cause the solute concentration in the droplets to be different from that of the nebulized fluid (80, 85).

1.4.2. Mechanism of Drug Deposition

Drugs for inhalation therapy are administered in aerosol form. The ability of the aerosolized drug to reach the peripheral airways is a prerequisite for efficacy. The regional pattern of deposition efficiency determines the specific pathways and rate at which deposited particles are ultimately cleared and redistributed (86). The pathology of disease of the lungs may considerably affect aerosol deposition. Patients with airway obstruction (eg, emphysema, asthma, chronic bronchitis) who inhaled radiolabeled aerosol showed increased central (tracheobronchial) deposition and diminished penetration to the peripheral pulmonary regions (87). The mechanisms by which particles deposit in the respiratory tract include impaction (inertial deposition), sedimentation (gravitational deposition), brownian diffusion, interception, and electrostatic precipitation (86, 88, 89). The relative contribution of each depends on the characteristics of the inhaled particles, as well as on breathing patterns and respiratory tract anatomy. All mechanisms act simultaneously, but the first two mechanisms are most important for large-particle deposition within the airways (1 mm , MMAD , 10 mm).

Diffusion, however, is the main determinant of deposition of smaller particles in peripheral regions of the lung (90).

Impaction occurs when a particle's momentum prevents it from changing course in an area where there is a change in the direction of bulk air flow. It is the main deposition mechanism in the upper airways, and at or near bronchial branching points. The probability of impaction increases with increasing air velocity, breathing frequency, and particle size (91, 86, 88).

Sedimentation results when the gravitational force acting on a particle overcomes the total force of the air resistance. Inspired particles will then fall out of the air stream at a constant rate (92). This is an important mechanism in small airways having low air velocity. The probability of sedimentation is proportional to residence time in the airway and to particle size, and decreases with increasing breathing rate.

Diffusion occur when the collision of gas molecules with small aerosol particles exerts discrete non-uniform pressures at the particles' surfaces, resulting in random brownian motion. The effectiveness of brownian motion in depositing particles is inversely proportional to particle diameters of those particles, 0.5 μm, (93) and is important in bronchioles, alveoli, and at bronchial airway bifurcations. Molecule-size particles may deposit by diffusion in the upper respiratory tract, trachea, and larger bronchi.

1.4.3. Factors Controlling Respiratory Drug Deposition

The factors that control drug deposition are:

(1) characteristics of the inhaled particles, such as size, distribution, shape, electrical charge, density, and hygroscopicity,

(2) anatomy of the respiratory tract, and

(3) breathing patterns, such as frequency, tidal volume, and flow.

Of these factors, aerosol particle size and size distribution are the most influential on aerosol deposition.

The size of the particles is a critical factor affecting the site of their deposition, since it determines operating mechanisms and extent of penetration into the lungs (94). Aerosol size is often expressed in terms of aerodynamic diameter (AD). The aerodynamic diameter is defined as the equivalent diameter of a spherical particle of unit density having the same settling velocity from an air stream as the particle in question (91). Thus, particles that have higher than unit density will have actual diameters smaller than their AD. Conversely, particles with smaller than unit density will have geometric diameters larger than their AD.

Aerosol size distributions may be characterized as practically monodisperse (uniform sizes) or polydisperse (nonuniform sizes).

The upper airways (nose, mouth, larynx, and pharynx) and the branching anatomy of the tracheobronchial tree act as a series of filters for inhaled particles. Thus, aerosol particles bigger than 100 µm generally do not enter the respiratory tract and are trapped in the naso/oropharynx. Particles bigger than 10 µm will not penetrate the tracheobronchial tree. Particles must generally be smaller than 5 µm in order to reach the alveolar space (92, 94, 95). On the other hand, particles smaller than 0.5 µm in diameter penetrate the lung deeply, but have a high tendency to be exhaled without deposition.

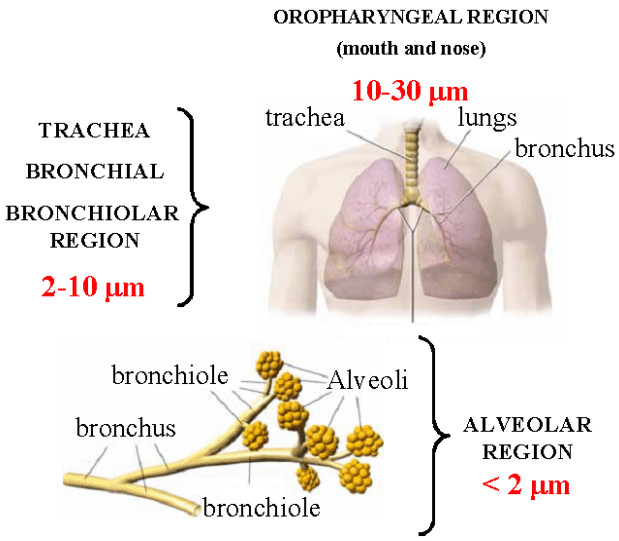


Figure 1.5: Particle Size And Sites Of Their Deposition

1.4.4. Respiratory Tract Anatomy

Airway geometry affects particle deposition in various ways. For example, the diameter sets the necessary displacement by the particle before it contacts an airway surface, cross-section determines the air velocity for a given flow, and variations in diameter and branching patterns affect mixing between tidal and reserve air (91). In contrast to many species of laboratory animal, humans have large lungs, a more symmetrical upper bronchial airway pattern, and are not obligate nose breathers. These anatomical differences produce greater amounts of upper bronchial particle deposition in humans (96).

1.4.5. Respiratory Patterns

The pattern of respiration during aerosol exposure influences regional deposition, since breathing volume and frequency determine the mean flow rates in each region of the respiratory tract, which, in turn, influence the effectiveness of each deposition mechanism (91, 97, 98, 99). Turbulence tends to enhance particle deposition, the degree of potentiation depending on the particle size. Rapid breathing is often associated with increased deposition of larger particles in the upper respiratory tract, while slow, steady inhalation increases the number of particles that penetrate to the peripheral parts of the lungs (100). Slow breathing, with or without breath-holding, showed a broad maximum deposition in the ciliated airways (tracheobronchial region). The pulmonary maximum occurred between 1.5 μm and 2.5 μm with breath-holding and between 2.5 μm and 4μm without breath-holding. Rapid inhalation showed similar trends: the tracheo-bronchial region maximum falls and shifts to between 3 μm and 6 μm. Pulmonary deposition sharpens and occurs between 1.5 μm and 2 μm with breath-holding, and between 2 μm and 3 μm without breathholding. When the above considerations are taken into account, the ideal scenario for aerosol would be: (1) aerosol AD smaller than 5 μm, to minimize oropharyngeal deposition, (2) slow, steady inhalation, and (3) a period of breath-holding on completion of inhalation.

1.4.6. Pulmonary Clearance

The primary function of the pulmonary defensive response to inhaled particles is to keep the respiratory surfaces of the alveoli clean and available for respiration. The elimination of particles deposited in the lower respiratory tract serves an important defence mechanism to prevent potentially adverse interactions of aerosols with lung cells. Insoluble particulates are cleared by several pathways, which are only partially understood. These pathways are known to be impaired in certain diseases and are thought to depend on the nature of the administered material (101, 102). Swallowing, expectoration, and coughing constitute the first sequence of clearance mechanisms operating in the naso/oropharynx and tracheobronchial tree.

A major clearance mechanism for inhaled particulate matter deposited in the conducting airways is the mucociliary escalator, whereas uptake by alveolar macrophages (86, 103) predominates in the alveolar region. In addition to these pathways, soluble particles can also be cleared by dissolution with subsequent absorption from the lower airways. The rate of particle clearance from these regions differs significantly and its prolongation can have serious consequences, causing lung diseases from the toxic effects of inhaled compounds. It is now well recognized that the lungs are a site for the uptake, accumulation, and/or metabolism

of numerous endogenous or exogenous compounds. All metabolizing enzymes found in the liver are also found in the lung, although in smaller amounts. The rate at which a drug is cleared and absorbed from the respiratory tract depends on the dynamic interaction of several factors, predominantly: (1) the mucociliary clearance rate, (2) site of deposition along the airways, (3) biopharmaceutical factors (particulates vs drug in solution), (4) drug release rate, and (5) the physicochemical properties of the drug, such as molecular weight, partition coefficient, and charge.

1.4.7. Mucociliary Clearance

Mucociliary clearance is a physiologic function of the respiratory tract to clear locally produced debris, excessive secretions, or unwanted inhaled particles. It consists of ciliated epithelial cells reaching from the naso/oropharynx and the upper tracheobronchial region down to the most peripheral terminal bronchioles. Beating of the cilia, together with mucus secreted by the goblet cells, contributes to an efficient clearance mechanism.

For normal mucociliary clearance to occur it is necessary that the epithelial cells are intact, the ciliary activity and the rheology of mucus is normal, and that the depth and chemical composition of the periciliary fluid layer is optimal. Thus, the mucociliary escalator can be impaired by altering the volume of mucus secretion, the mucus viscosity and elasticity, or the ciliary beat frequency. Mucociliary clearance is known to be impaired in smokers (104), in patients with chronic bronchitis (102), and in acute asthmatics (105). Certain diseases have the opposite effect that of enhancing clearance rates (106).

1.5. Rifampicin

Each year, there are 8–10 million new cases of TB, which is the leading cause of death in adults by an infectious agent (107, 108).

Rifampicin (RFP), 3-(4-methyl-l-piperazinyl-irninomethyl) rifamycin (109, 110) is one of the most potent and broad spectrum antibiotics against bacterial pathogens and is a key component of anti-TB therapy. The introduction of RFP in 1968 greatly shortened the duration of TB chemotherapy. RFP diffuses freely into tissues, living cells, and bacteria, making it extremely effective against intracellular pathogens like M. tuberculosis (108). However, bacteria develop resistance to RFP with high frequency, which has led the medical community in the United States to commit to a voluntary restriction of its use for treatment of TB or emergencies.

RFP is an amphiphilic compound. It is extensively recycled in the enterohepatic circulation, and metabolites formed by deacetylation in the liver are eventually excreted in the faeces.

Formula: C43H58N4O12;

Molecular Weight: 822.95.

Log P: 4,24

pKa 1: 1,7

pKa 2: 7,9

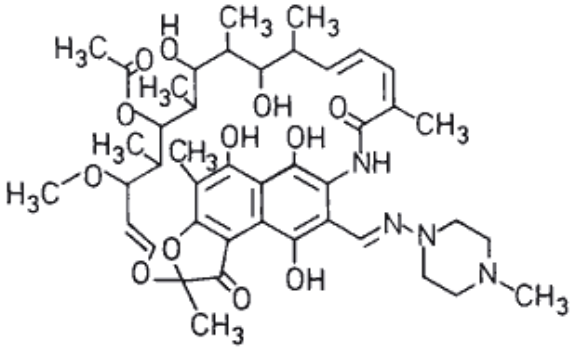


Figure 1.6: Chemical Structure of Rifampicin.

The bactericidal activity of RFP stems from its high affinity binding to, and inhibition of, the bacterial DNA-dependent RNA polymerase (RNAp) (111). The essential catalytic core RNAp of bacteria (subunit composition $\alpha_2\beta\beta'\varpi$) has a molecular mass of around 400 kDa and is evolutionarily conserved among all cellular organisms (112).

Mutations conferring RFP resistance map almost exclusively to the rpoB gene (encoding the RNAp β-subunit) in every organism tested, including E. coli (113, 114, 115) and M. tuberculosis (116, 117). Comprehensive genetic analyses have provided molecular details of amino acid alterations in β conferring RFP resistance (118, 119, 120, 121, 122, 123).

Variable bioavailability of RFP from separate as well as fixed dose combination formulations has been perceived as a major bottleneck in successful treatment of TB (124). In each formulations important factor that may affect rate and/or extent of dissolution is physical characteristics of rifampicin raw material such as polymorphic form, particle size, etc., which in turn have a direct impact on drug substance processability, drug product manufacturability, and quality of dosage forms, including stability, dissolution, and bioavailability (125). Therefore, a complete physical characterization and its biopharmaceutic implication is essential in order to determine the influence of polymorphism/physical form on bioavailability. RFP due to its complex structure exhibits polymorphism and exists in two polymorphic forms (126). It also exists as hydrates and various solvates, which eventually convert into amorphous form at room temperature or after desolvation (127).

RFP is well tolerated by most patients at currently recommended doses, although gastrointestinal tolerance can be unacceptably severe. Other adverse effects (skin rashes, fever, influenza-like syndrome and thrombocytopenia) are more likely to occur with

intermittent administration. Exfoliative dermatitis is more frequent in HIV-positive TB patients. Temporary oliguria, dyspnoea and haemolytic anaemia have also been reported in patients taking the drug three times weekly. These reactions usually subside if the regimen is changed to one with daily dosage. However, dose-related hepatitis can occur which is potentially fatal.

RFP induces hepatic enzymes, and may increase the dosage requirements of drugs metabolized in the liver. These include corticosteroids, steroid contraceptives, oral hypoglycaemic agents, oral anticoagulants, phenytoin, cimetidine, cyclosporin and digitalis glycosides.

1.6. Microspheres as Controlled Delivery Systems

Drug delivery systems (DDS) that can precisely control the release rates or target drugs to a specific

body site have had an enormous impact on the healthcare system. The last two decades in the pharmaceutical industry have witnessed an avant-garde interaction among the fields of polymer and material science, resulting in the development of novel drug delivery systems (128). Carrier technology offers an intelligent approach for drug delivery by coupling the drug to a carrier particle such as microspheres, nanoparticles, liposomes, etc. which modulates the release and absorption characteristics of the drug.

Conventional drug administration does not usually provide rate-controlled release or target specificity. In many cases, conventional drug delivery provides sharp increases of drug concentration at potentially toxic levels. Following a relatively short period at the therapeutic level, drug concentration eventually drops off until re-administration.

Today new methods of drug delivery are possible: desired drug release can be provided by rate-controlling membranes or by implanted biodegradable polymers containing dispersed medication. Over the past 25 years much research has also been focused on degradable polymer microspheres for drug delivery. Microspheres constitute an important part of these particulate DDS by virtue of their small size and efficient carrier characteristics. Administration of medication via such systems is advantageous because microspheres can be ingested, injected or inhaled; they can be tailored for desired release profiles and in some cases can even provide organ-targeted release. The idea of controlled release from polymers dates back to the 1960s through the employment of silicone rubber (129) and polyethylene (130). The lack of degradability in these systems implies the requirement of eventual surgical

removal and limits their applicability. In the 1970s biodegradable polymers were suggested as appropriate drug delivery materials circumventing the requirement of removal (131). The idea of polymer microspheres as delivery systems was reported as early as the 1960s (132) and degradation was incorporated by Mason et al. (133) through the employment of a degradable polymer.

Recent literature shows that suspensions of degradable microspheres can be employed for sustained drug release at desirable doses. Biocompatibility can be achieved by the use of natural polymers such as cellulose, chitin, and chitosan or by the employment of semisyntetic polymers made from naturally occurring monomers such as lactic and glycolic acids. Polymers derived from synthetic monomers also show excellent delivery properties. However, their toxicity effects may require evaluation. The factors affecting drug release are controllable; they are attributed to properties such as polymer molecular weight, as well as microsphere size, distribution, morphology and make-up.

For preparation of microspheres using biodegradable polymers, it is important to choose an appropriate encapsulation process which meets the following requirements. First, the chemical stability and biological activity of the incorporated drugs should be maintained during the encapsulation process. For example, since most proteins are readily denatured upon contact with hydrophobic organic solvents or acidic/basic aqueous solutions, the process should avoid such harsh

environments. Second, the encapsulation efficiency and the yield of the microparticles should be high enough for mass production. Third, the microparticles produced should have the reasonable size range. Fourth, the release profile of the drug should be reproducible without the significant initial burst. Fifth, the process employed should produce free-flowing microparticles, thus making it easy to prepare uniform suspension of the microparticles. There are a number of techniques available for microencapsulation of drugs such as the emulsion-solvent evaporation/extraction method, spray drying, phase separation-coacervation, interfacial deposition, precipitation method, in situ polymerization, etc. Each method has its own advantages and disadvantages. The choice of a particular technique depends on the attributes of the polymer and the drug, the site of the drug action, and the duration of the therapy (134, 135, 136).

Increasing or controlling the encapsulation efficiency (E%) is desirable, it can prevent the loss of precious medication and it can help to extend the duration and dosage of treatment. Yang et al. (137) have provided a revealing study which correlated the encapsulation efficiency to

sphere preparation temperature. The authors found that the highest encapsulation efficiencies occurred at the lowest and highest formation temperatures tested (about 50% at 4 and 38°C, and about 19% at 22 and 29°C). The non-linear drug loading trend suggested that different mechanisms governed the encapsulation process at different temperatures. When considering the relation of the polymer itself to encapsulation efficiency, Ghaderi et al. (138) found that increasing the concentration of polymer in the organic phase increased the encapsulation efficiency. An increase in E% from 1 to 25% was observed depending on the concentration of the polymer.

Microsphere size can be affected by the polymer concentration, temperature, viscosity, the stirring rate, and the amount of emulsifier employed. Considering the effect of polymer concentration, it has often been reported that increasing the concentration of polymer increases sphere size (138, 139, 140, 141, 142). Yang et al. (137) used scanning electron microscopy (SEM) to show that sphere size was temperature dependent; lower and higher temperatures produced larger spheres whereas intermediate temperatures produced smaller spheres. Once again, different mechanisms dominated microsphere formation at different temperatures. At lower temperatures, the solution's higher viscosity resulted in the formation of larger spheres; this has also been confirmed by other researchers (143). Larger spheres were obtained at higher temperatures due to the higher rate of solvent evaporation which resulted in higher solvent flow pressure moving more material from the sphere center outward (137).

Jalil and Nixon (144) studied the variation of sphere size with respect to the stirring rate and the influence of the emulsifier in the second emulsion step. It was shown that microsphere size decreased with increasing stirring rate since increased stirring results in the formation of finer emulsions. Little change in diameter size was reported by varying emulsifier concentration.

Controlled release is an attainable and desirable characteristic for DDS. The factors affecting the drug release rate revolve around the structure of the matrix where the drug is contained and the chemical properties associated with both the polymer and the drug. Conventional oral delivery is not rate controlled. A drug encapsulated in a slowly degrading matrix provides the opportunity for slower release effects, but polymer degradation is not the only mechanism for the release of a drug. The drug release is also diffusion controlled as the drug can travel through the pores formed during sphere hardening. In some cases, drugs containing nucleophilic groups can cause increased chain scission of the polymer matrix, which also

increases the rate of drug expulsion. Polymer molecular weight, drug distribution, polymer blending, crystallinity, and other factors are important in manipulating release profiles. The most desirable release profile would show a constant release rate with time. However, in many cases release profiles are more complicated and often contain two main expulsion processes: the first being an initial burst of expelled medication from the sphere surface; the second, a usually more constant stage with release rates dependent on diffusion and degradation (145, 146, 147).

The release profiles are also dependent on the size of the microspheres; the rate of drug release was found to decrease with increasing sphere size (148, 149, 150, 151, 152). Therefore, by mixing microspheres of different sizes it is possible to obtain another degree of controlling release. More importantly, linear, zero-order kinetics are obtainable by combining the proper formulation of microsphere sizes.

Core-shell microspheres usually refer to spheres formed by making core units through a normal preparative method, followed by the addition of an outer layer by a dipping procedure, mixing procedure, or emulsion procedure (153, 154, 155, 156, 157, 158). Employment of a shell is usually meant to enhance controlled release and possibly reduce the effect of the initial burst.

1.7. Bioadhesive Microspheres as Systems Able to Enhance Pulmonary Drug Delivery

The success of microspheres as DDS is limited due to their short residence time at the site of absorption. It would, therefore, be advantageous to have means for providing an intimate contact of the DDS with the absorbing membranes. It can be achieved by coupling bioadhesion characteristics to microspheres and developing novel delivery systems referred to as “bioadhesive microspheres”.

“Bioadhesion” in simple terms can be described as the attachment of a synthetic or biological macromolecule to a biological tissue. An adhesive bond may form with either the epithelial cell layer, the continuous mucus layer or a combination of the two. The term “mucoadhesion” is used specifically when the bond involves mucous coating and an adhesive polymeric device, while “cytoadhesion” is the cell-specific bioadhesion. Bioadhesive microspheres include microparticles and microcapsules (having a core of the drug) of 1–1000 μm in diameter and consisting either entirely of a bioadhesive polymer or having an outer coating of

it, respectively (159). Microspheres, in general, have the potential to be used for targeted and controlled release drug delivery; but coupling of bioadhesive properties to microspheres has additional advantages, e.g. efficient absorption and enhanced bioavailability of the drugs due to a high surface to volume ratio, a much more intimate contact with the mucus layer, specific targeting of drugs to the absorption site achieved by anchoring plant lectins, bacterial adhesins and antibodies, etc. on the surface of the microspheres. Microspheres prepared with bioadhesive and bioerodible polymers undergo selective uptake by the macrophages cells in lung mucosa and by the M cells of Peyer patches in gastrointestinal (GI) mucosa. Bioadhesive microspheres offer unique carrier system for many pharmaceuticals and can be tailored to adhere to any mucosal tissue, including those found in eyes, oral cavity and throughout the respiratory, urinary and gastrointestinal tract.

Increased residence time of particulate delivery systems at the mucosal surface may facilitate the increased uptake of such particlres. To this end, muco- or bioadhesive agents provide a strategy that may help to increase the residence time and, hence, the uptake of biodegradable particulate when administered by the pulmonary routes.

Agents such as hydroxypropylcellulose (HPC) (160, 161), chitosan (162), carbopol (163), carboxymethylcellulose, hyaluronic acid and polyacrylic acid (164) have all shown promise as muco/bio-adhesive agents for potential use in pulmonary delivery, either alone, in combination with another carrier or incorporated into the structure of the carrier itself (165).

1.8. Microspheres for Inhalation

Aerosolised administration of drugs to the lung has been employed for many years to treat primarily localised disease states within the bronchi. Since this route of administration can deliver therapeutic agents to the diseased regions whilst reducing their distribution to the other organs, it provides an excellent example of targeted drug therapy. Hence, a more favourable therapeutic index can be obtained for the treatment of lung diseases when drugs are administered by inhalation rather than by the oral route. Bronchodilators, anti-inflammatory agents, mucolytics, antiviral agents, anticancer agents and phospholipidprotein mixtures for surfactant replacement therapy are all routinely given as aerosolised formulations whilst more recently, there has been an increasing interest in the delivery of drugs via the lung to treat pulmonary deseases in particular these associated with AIDS. Moreover, the development of potent protein drugs by biotechnology has also stimulated a growth of interest in inhalation aerosols because of the possibility of systemic delivery of these drugs via the airways (166).

A significant disadvantage of many existing inhaled drugs is the relatively short duration of resultant clinical effects and most medications in aerosol form require inhalation at least 3-4 times daily (167). This often leads to poor patient compliance with the therapeutic regime and increases the possibility of associated side effects due to the risk of self-administration of the drug by the patients. A reduction in the frequency of dosing would be convenient, particularly for chronic treatments such as those for asthma. Sustained release of such drugs in the lung would be particularly beneficial since they could be delivered to and retained at the targeted receptors for a prolonged period of time and thus minimise the biodistribution throughout the systemic circulation.

The potential advantages of achieving sustained release to the lung has been shown by the improved therapeutic effects obtained with a corticosteroid inhaled four times a day compared to two times a day (168). Controlled release of drugs within the pulmonary tree also offers many distinct advantages for agents which are administered for systemic actions. Many of these, in the future, are likely to be potent proteins and peptides designed to regulate important biological responses (169) and the pulmonary route provides many potential advantages compared to other portals of delivery. Currently, a number of methods have been investigated as potential pulmonary sustained-release systems for short-acting drugs. These include the incorporation of drugs in liposomes and in particular in biodegradable microspheres.

The use of controlled release polymeric systems is an approach that holds promise for improving the duration and effectiveness of inhaled drugs, for both local and systemic action (170).

Initial studies with polymeric aerosol systems showed that properly engineered, large porous particles (LPP) were also capable of delivering bioactive insulin to the blood of rats and control glucose levels for 96 h. The previous longest sustained delivery of insulin to the blood via the lungs was only 6 h, using liposomes that were intratracheally instilled into rat lungs. Since then, only limited examples of polymeric aerosol systems have been reported. For example, cationic polymers, such as polyethyleneimine (PEI) and poly-l-lysine (PLL), complexed with DNA have also been tested in the airways as a method to achieve transient gene expression.

For example albumin microspheres can be prepared by either physical denaturation or chemical cross-linking of albumin droplets. The role of albumin microspheres as drug delivery systems for targeted and sustained release after intravenous administration has been

the subject of extensive research. Some of these studies have been conducted with the intention of targeting drugs selectively to the lung (171). However, the biodegradability, lack of toxicity and immunogenicity, ready availability and capability to undergo chemical modification could render them suitable as a carrier for inhalation of drugs. The possibility of using drug-containing albumin microspheres (172) as an inhaled dry powder was also investigated, employing similar preparation and in vitro evaluation procedures (173). Tetrandrine, an antisilicotic alkaloid, was entrapped in albumin microspheres and factorial design employed to optimise particle size and drug entrapment. The tetrandrine recovered from the lower stage of a twin-stage liquid impinger operated under the pharmacopoeial conditions was 13.83 ± 2.58% (n = 6) and such levels were considered sufficient for therapeutic efficiency. Thus, albumin microspheres have the potential to deliver tetrandrine to the alveolar region where they may be metabolised to incorporate the drug in alveolar macrophages, which are thought to be the main site of action of tetrandrine.

Also PGL microspheres as a successful drug delivery system, have been used for targeting and controlled release of a wide range of drugs, including peptides and proteins (174). Their potential use in pulmonary delivery has also been explored. Masinde and Hickey (175) were able to prepare poly(lactic acid) (PLA) microspheres with particle sizes between 1 and 11μm by a solvent evaporation technique. After suspending the microspheres in a non-surfactant solution this was subsequently atomised by a jet nebulizer, particles were generated which were suitable for drug delivery to the lower airways, having a median diameter of 2 μm and geometric standard deviation of 2.4/zm. Sustained bronchodilation was reported by Lai et al. (176) after PGL microspheres with a mean diameter of 4.5/zm containing entrapped isoproterenol (7% w/w) were intratracheally administered to Long-Evans rats. Even though 70% of the incorporated isoproterenol had been released from the MS into the instillation medium prior to administration, the drug still significantly ameliorated serotonin-induced bronchoconstriction for more than 12 h at a dose of 0.1 mg kg -1.

The use of polymeric microparticles to deliver anti-tubercular drugs (ATDs) by different routes (injectable, oral and aerosol) has been reported by several investigators. In recent years, one of the best ways to achieve higher drug levels in the lungs has been the development of new formulations (microparticle-based) that are directly delivered to the lungs via the aerosol route.

For example, several groups have investigated respiratory delivery of microsphere-encapsulated antibiotics for the local treatment of tuberculosis (177, 178). It has been

observed that particles reaching the lungs are phagocytosed rapidly by alveolar macrophages. Although phagocytosis and sequestration of inhaled powders may be a problem for drug delivery to other cells comprising lung tissue, it is an advantage for chemotherapy of TB. Phagocytosed microparticles potentially can deliver larger amounts of drug to the cytosol than oral doses. Moreover, microparticles have the potential for lowering dose frequency and magnitude, which is especially advantageous for maintaining drug concentrations and improving patient compliance. It may therefore be advantageous to incorporate inhalable microparticles containing multiple drugs in a inhalation system for chemotherapy of TB.

Because of its biodegradability and biocompatibility, poly (lactide-co-glycolide) (PLGA; a synthetic polymer) has been a popular choice as a drug carrier. Patrick O'Hara et all. by employing solvent evaporation as well as spray drying methods, PLGA microparticles encapsulating rifampicin were prepared (179). The microspheres were administered via insufflation or nebulization to guinea pigs, 24 h before aerosol infection with M. tuberculosis H37Rv. The model was adopted as a post-treatment screening method for antimicrobial efficacy. The assessment of colony forming units (cfu) 28 days post-infection showed a dose–effect relationship, i.e. lower cfu with higher doses of microspheres. The cfu count was significantly reduced compared with free rifampicin. With a similar experimental approach, the authors next evaluated the effect of repeated dosing of the microspheres. At 10 days post-infection, half of the treatment group received a second dose of the microspheres. There was a significant reduction in cfu in lungs (but not in spleens) in the case of animals receiving a single dose of the formulation, whereas two doses resulted in a significant decrease in cfu in lungs as well as in spleens. It was realized that besides the methodology involved in microparticle preparation, the surface characteristics of dry powders also play a key role in predicting particle dispersion and pulmonary deposition. Although the results with rifampicin-loaded microspheres proved to be encouraging, it was necessary to incorporate other ATDs because the disease requires multidrug therapy for its cure. Hence, other investigators encapsulated isoniazid with rifampicin in polylactide microparticles for dry powder inhalation to rats. Drug concentrations inside the alveolar macrophages were found to be higher than that resulting from systemic delivery of free drugs, an indication of the rapid phagocytic uptake and cytosolic localization of the drug-loaded microparticles. The authors discussed that since alveolar macrophages migrate to secondary lymphoid organs, loading these cells with microparticles might lead to transport of drugs to those sites where macrophages migrate (mimicking the course of spread of mycobacteria).

A. zahoor et al. developped a natural polymer-based inhalable drug delivery system to overcome the limitations associated with various drug delivery systems. Sodium alginate, a natural polymer with properties such as an aqueous matrix environment, high gel porosity and biocompatibility, and approved by the US Food and Drug Administration (FDA) for oral use (180, 181), was used to prepare particles encapsulating three antitubercular drugs (ATDs). Alginate microparticles containing isoniazid (INH), pyrazinamide (PZA) and rifampicin (RIF) were developed and characterised, and pharmacokinetic and pharmacodynamic evaluation was carried out via the aerosol route in guinea pigs. The majority of particles (80.5%) were in the respirable range, with mass median aerodynamic diameter of 1.1±0.4μm and geometric standard deviation of 1.71±0.1μm. The relative bioavailabilities of all drugs encapsulated in alginate particles were significantly higher compared with oral free drugs. All drugs were detected in organs (lungs, liver and spleen) above the minimum inhibitory concentration until 15 days post nebulization, whilst free drugs stayed up to day 1. The chemotherapeutic efficacy of three doses of drug-loaded alginate nanoparticles nebulised 15 days apart was comparable with 45 daily doses of oral free drugs. Thus, inhalable alginate particles can serve as an ideal carrier for the controlled release of antitubercular drugs.

The rising incidence of multidrug-resistant TB (MDR-TB) is a matter of great concern because the treatment involves the use of second-line ATDs, which are more costly and toxic compared with the first-line drugs used to treat drug-susceptible TB. Furthermore, the treatment schedule is more prolonged with a greater risk of patient non-compliance (182). Some of the second-line drugs, e.g. para-aminosalicylic acid (PAS), need to be administered in very large amounts (up to 12 g daily), which is inconvenient to the patient. In order to reduce the drug dosage, investigators have formulated an inhalable microparticulate system for PAS, based on dipalmitoylglycero-3-phosphocholine. The microparticles were produced by spray drying, possessed a 95% drug loading and were administered to rats via insufflation. The drug was maintained at therapeutic concentrations in the lung tissue for at least 3 h (the authors did not monitor the drug levels further) following a single dose of just 5mg of the dried formulation. Accelerated stability studies indicated that the formulation was stable for up to 4 weeks.

Properly designed new polymeric aerosols, with the ability to target various regions of the lung, should prove beneficial for prolonged non-invasive treatment of both lung disorders, such as asthma, cystic fibrosis, mycobacteriosi and diseases requiring drug delivery to the systemic circulation.

2. Chitosan and PLGA: General Informations

2.1. Chitosan and Chitosan Microspheres as Controlled Delivery System

Chitosan, a natural linear biopolyaminosaccharide, is obtained by alkaline deacetylation of chitin, which is the second abundant polysaccharide next to cellulose (183, 184). Chitin is the principal component of protective cuticles of crustaceans such as crabs, shrimps, prawns, lobsters and cell walls of some fungi such as aspergillus and mucor. Chitin is a straight homopolymer composed of β-(1,4)-linked N-acetyl-glucosamine units while chitosan comprises of copolymers of glucosamine and N-acetyl-glucosamine (185, 186, 187). Chitosan has one primary amino and two free hydroxyl groups for each C_6 building unit.

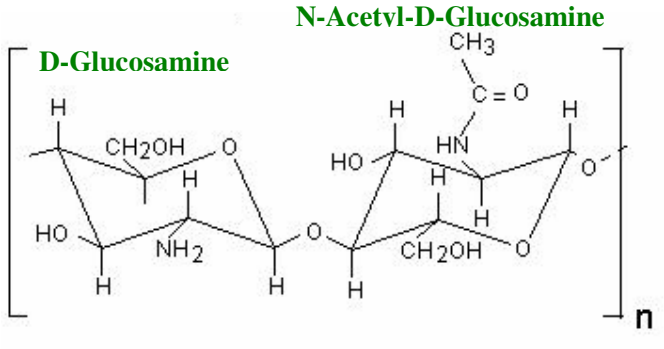


Figure 2.1: Chitosan Chemical Structure

Due to the easy availability of free amino groups in chitosan, it carries a positive charge and thus in turn reacts with many negatively charged surfaces/polymers and also undergoes chelation with metal ions (188). Chitosan is a weak base and is insoluble in water and organic solvents, however, it is soluble in dilute aqueous acidic solution (pH < 6.5), which can convert the glucosamine units into a soluble form ($R–NH_3^+$) (189). It gets precipitated in alkaline solution or with polyanions and forms gel at lower pH. Commercially, chitosan is available in the form of dry flakes, solution and fine powder. It has an average molecular weight ranging between 3800 and 2,000,000 and is from 66 to 95% deacetylated (181). Particle size, density, viscosity, degree of deacetylation, and molecular weight are important characteristics of chitosan which influence the properties of pharmaceutical formulations based on chitosan. Properties such as biodegradability, low toxicity and good biocompatibility make it suitable for use in biomedical and pharmaceutical formulations (189, 190), e.g. it is

used for hypobilirubinaemic and hypocholesterolemic effects (191, 192), antiacid and antiulcer activities, wound and burn healing properties (193), immobilization of enzymes and living cell and in ophthalmology (194). Among pharmaceutical applications it has been used as a vehicle for directly compressed tablets (195, 196, 197), as a disintegrant (197), as a binder (198), as a granulating agent (199), in ground mixtures (200), as a drug carrier for sustained release preparations (201, 202, 203, 204) as well as a co-grinding diluent for the enhancement of dissolution rate and bioavailability of water insoluble drugs (205, 206, 207). Chitosan has been shown to possess mucoadhesive properties (208, 209, 210) due to molecular attractive forces formed by electrostatic interaction between positively charged chitosan and negatively charged mucosal surfaces. These properties may be attributed to: (a) strong hydrogen bonding groups like –OH, –COOH (211); (b) strong charges (212); (c) high molecular weight (213); (d) sufficient chain flexibility (209); and (e) surface energy properties favoring spreading into mucus (214). The positive charge on chitosan polymer gives rise to strong electrostatic interaction with mucus or negatively charged sialic acid residues on the mucosal surface. Chitosan also shows good bioadhesive characteristics and can reduce the rate of clearance of drug from the pulmonary system thereby increasing the bioavailability of drugs incorporated in it (215).

Chitosan possess suitable properties as a carrier for microsphere drug delivery. Chitosan microspheres are the most widely studied drug delivery systems for the controlled release of drugs, antibiotics, antihypertensive agents, anticancer agents, proteins, peptide drugs and vaccines.

Chitosan microspheres are used to provide controlled release of many drugs and to improve the bioavailability of degradable substances such as protein or enhance the uptake of hydrophilic substances across the epithelial layers. Chitosan has also been used as a potential carrier for prolonged delivery of drugs, macromolecules and targeted drug delivery. Magnetic chitosan microspheres used in targeted drug delivery are expected to be retained at the target site capillaries under the influence of an external magnetic field (216). Also, strong interaction between cationic microspheres and anionic glycosaminoglycan receptors can retain the microspheres in the capillary region (216).

Reacting chitosan with controlled amounts of multivalent anion results in crosslinking between chitosan molecules. The crosslinking may be achieved in acidic, neutral or basic environments depending on the applied method. This crosslinking has been extensively used

for the preparation of chitosan microspheres. Processes used for the preparation of the microspheres are shown in Figure 2.

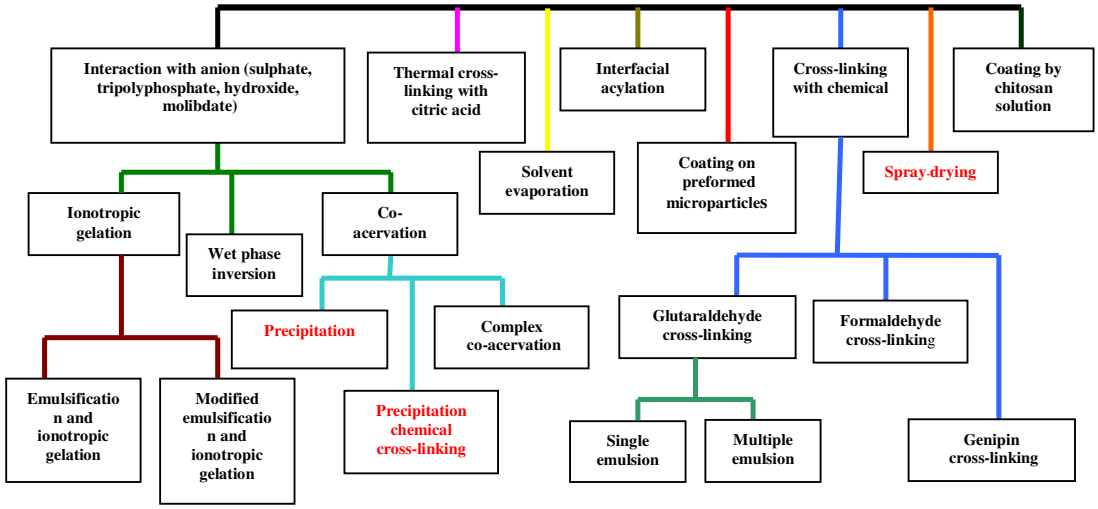


Figure 2.2: Chitosan Microspheres: Preparation Methods

The entrapment efficiency of the drugs in chitosan microspheres can be affected by many factors, e.g. nature of the drug, chitosan concentration, drug-polymer ratio, stirring speed, etc. Generally a low concentration of chitosan shows low encapsulation efficiency (217). However, at higher concentrations, chitosan forms highly viscous solutions, which are difficult to process. A number of reports have shown that entrapment efficiency increases with an increase in chitosan concentration. A study carried out by Nishioka et al. (218) also revealed that the cisplatin content increased with increasing chitosan concentration. Microspheres made with a mixture of high molecular weight/low molecular weight chitosan (1:2 w/w) showed good drug content and encapsulation efficiency and these were independent of polymer/drug ratio.

In an attempt to incorporate the drug onto previously formed chitosan microspheres, prednisolone sodium phosphate was adsorbed to previously manufactured chitosan microspheres (219, 220). The drug adsorption was found to be dependent upon the initial drug concentration. A higher initial concentration led to a higher loading efficiency. It was also observed that lipophilic steroids were adsorbed in lower amounts as compared to their hydrophilic derivatives. Hejazi and Amiji (221) prepared chitosan microspheres by ionic crosslinking and precipitation with sodium sulfate. Two different methods were used for drug

loading. In first method, tetracycline was mixed with chitosan solution before the simultaneous crosslinking and precipitation. In second method, the drug was incubated with pre-formed microspheres for 48 h. When the drug was added to the polymer solution before crosslinking and precipitation, only 8% (w/w) was optimally incorporated in the final microsphere formulation. On the other hand, when the drug was incubated with the pre-formed microspheres, a maximum of 69% (w/w) could be loaded. This signifies that the drug can be adsorbed on the chitosan microspheres to a greater extent using the latter method.

Many parameters determine the drug release behavior from chitosan microspheres. These include concentration and molecular weight of the chitosan, the type and concentration of crosslinking agent, variables like stirring speed, type of oil, additives, crosslinking process used, drug-chitosan ratio, etc.

Drug release studies from chitosan microspheres have generally shown that the release of the drug decreases with an increase in molecular weight of chitosan. This may be attributed to swelling behavior of chitosan microspheres. An increase in molecular weight of chitosan leads to increase in viscosity of the gel layer, which influences the drug diffusion as well as erosion of the microspheres. Increasing the polymer concentration drug release decreases.

A number of reports studying the effect of drug release have shown that the release of the drug from the microspheres increases with increase in drug content in the microspheres. However, different results have also been reported. Cross-linking density has a remarkable effect on the release of drugs from the microspheres. Jameela et al. (222) revealed that highly crosslinked microspheres released only 35% of the progesterone in 40 days compared to 70% release from microspheres cross-linked lightly. Kumar et al. (223) encapsulated curcumin (upto an extent of 79.49 and 39.66%) in bovine serum albumin and chitosan to form a depot forming drug delivery system. Microspheres were prepared by emulsion–solvent evaporation method coupled with chemical crosslinking of the natural polymers. The concentration of the crosslinking agent had remarkable influence on the drug release. In vitro release studies indicated a biphasic drug release pattern, characterized by a typical burst-effect followed by a slow release which continued for several days. Dini et al. (224) studied the synthesis and characterization of GA crosslinked chitosan microspheres containing hydrophilic drug, hydroquinone. It was found that slow drug release rates were obtained from microspheres prepared by using a high initial concentration of chitosan, a high molecular weight of chitosan or/and a low drug concentration. It was established that the release rate of hydroquinone was

mainly controlled by the polymer crosslinking density and the degree of swelling of the hydrogel matrix.

Chitosan microspheres are very stable. In fact, only few studies have reported the instability of chitosan microspheres (prepared by precipitation) in acidic medium. Berthold et al. (219) prepared chitosan microspheres by sodium sulfate precipitation but the acid stability of the microspheres was found to be poor. Lather, Berthold et al. (220) made an extensive investigation into the acid stability of the microspheres finding that cross-linking with GA can improve it.

Microparticles prepared using chitosan are being extensively investigated for various classes of drugs like anticancer drugs, antiinflammatory drugs, cardiac agents, antibiotics, antithrombotic agent, steroidal drugs, anticalcification agents, proteins, antigens, antidiabetic agents, growth factors, DNA encapsulation, diuretics, central nervous system (CNS) acting agents, anti-infective agents, gastrointestinal agents.

Chitosan is a versatile polymer whose applications range from weight supplement in the market to a drug carrier in formulation research.

2.2. PLGA and PLGA Microspheres as Controlled Delivery System

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 (226).

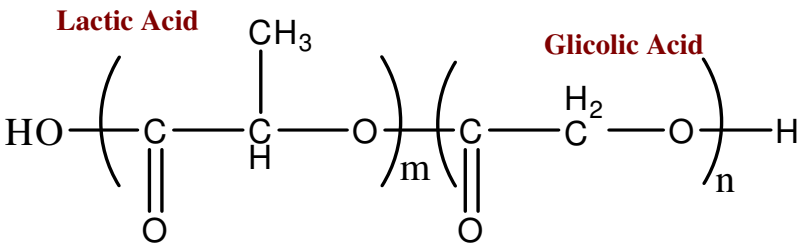


Figure 2.3: PLGA Chemical Structure

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 (227). Most importantly, PLA and PLGA have been approved by the United States Food and Drug Administration (FDA) for drug delivery.

The biodegradation of the PLGA occurs through random hydrolitic chain scissions of the swollen polymer. The cleavage of ester bond linkages yields carboxylic end groups and hydroxyl groups. The formed carboxylic groups then could catalyze and accelerate the hydrolysis of other ester bonds, a phenomenon referred as autocatalysis. The polymer erosion in delivery devices is the degradation of polymers to water-soluble fragments, accompanied by a progressive weight loss of the matrix. Generally, the polymer erosion could be classified into two mechanisms, namely surface or bulk erosion (228). In the case of surface erosion, the degradation is faster than the water diffusion. Thus the degradation and erosion take place on the surface of the matrix; in contrast, with bulk erosion, the water penetration is faster and the degradation and erosion affect all the polymer bulk. PLGA are bulk erosion polymers. The weight loss of the polymer devices doesn't take place at the beginning of the degradation of the PLGA. Accompanying with the produced water-soluble oligomers, significant weight loss occurs when the molecular weight of the PLGA reaches certain threshold (229). The heterogeneous degradation of the large size PLGA devices has been reported recently (230). It was found that after subcutaneously implantation, the molecular weight of the outer phase of the polymer plate was higher than that of the inner phase. The outer phase was solid but the inner phase was sometimes semisolid (230). During the degradation of the polyester, the formed soluble acidic oligomers inside the matrix may not easily diffuse out, which may lead to a more acidic microenvironment inside the matrix. Therefore the autocatalysis is more prominent in the bulk than at the surface, which leads to the surface-interior differentiation.

The physical and chemical characteristics of PLGA such as molecular weight, glass transition temperature, and copolymer ratios are crucial to the biodegradation behavior of the polymers.

At present, a numerous of analytical methodologies are introduced to characterize these properties, which then provide the potential clue to understand, predict and eventually modify the release behavior of the systems.

The most commonly used method to analyze the molecular weight of PLGA is the size exclusion chromatograph (SEC). Polydispersity reveals the molecular weight distribution. The higher the polydispersity, the wider the molecular weight distribution is (231).

Lactic acid contains an asymmetric carbon atom and has two optical isomers. PLA can exist in two stereo forms, optically active form (L-PLA) and optically inactive racemic form (D,LPLA). L-PLA is found to be semicrystalline in nature due to high regularity of its polymer chain while D,L-PLA is an amorphous polymer because of irregularities in its polymer chain structure. Hence the use of D,L-PLA is preferred over L-PLA as it enables more homogeneous dispersion of the drug in an optically inactive polymer matrix. Crystallinity of the PLGA can be determined by DSC or X-ray diffraction. It is directly related to the molecular weight, type, and molar ratio of the copolymer component. PLGAs prepared from L-PLA and PGA are crystalline copolymers while those from D,L-PLA and PGA are amorphous in nature. It was reported that PLGAs containing less than 70% glycolide are amorphous in nature (232).

Glass transition temperature (Tg) is the temperature at which the polymers change from glassy state to rubbery state. At this point, the mechanical behavior of the polymer changes from rigid and brittle to tough and leathery (plastic behavior). The Tg of PLGAs is commonly above the physiological temperature of 37 °C, which gives them enough mechanical strength to be fabricated into delivery devices. The Tg of the PLGA decreases with decrease of lactic acid content in copolymer and with decrease in their molecular weight (233).

Analysis of copolymer composition of PLGA can be accomplished by magnetic resonance spectroscopy (NMR).

The biodegradable profiles of PLGA could be influenced by the physical and chemical properties of the polymer and the additives or encapsulated drugs in the polymer matrix. In general, the degradation rate of the PLGA decreases with the decrease of 1) polymer molecular weight (234); 2) initial crystallinity (235); 3) lactic/glycolic copolymer ratio (236); 4) glass transition temperature (237); 5) hydrophilicity of the polymer

The degradation rate increase with incorporation of acidic or basic compounds (238)

Microparticles based on biodegradable polymer have been extensively investigated as controlled release delivery system over the past three decades. In recent years, a continued

interest in PLGA microparticles has been triggered by their application for the controlled release of macromolecular drugs. Biodegradable microparticles can be prepared by several methods, but the most widely used techniques are phase separation (coacervation), spray drying, and solvent evaporation.

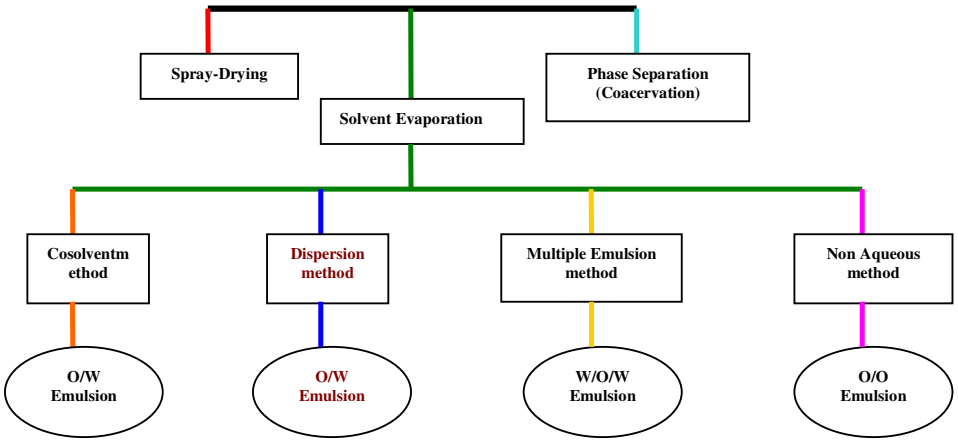


Figure 2.4: PLGA Microspheres: Preparation Methods

The manufacturing method has much influence on the structure and release properties of the microparticles.

General requirements for microparticle preparation include:

• Maintain the stability of the encapsulated active ingredient

• Obtain optimal drug loading, high encapsulation efficiency and yield

• Get desired drug release profiles and low initial release

• Involve a simple, reproducible, and scaleable process

Generally PLGA microspheres are prepared by using oil–water emulsions that consist of an organic phase comprised of a volatile solvent with dissolved polymer and the drug to be encapsulated, emulsified in an aqueous phase containing dissolved surfactant. Two common examples of volatile organic solvents used for the organic-phase solvent are dichloromethane and ethyl acetate. A surfactant is also included in the aqueous phase to prevent the organic droplets from coalescing once they are formed. Once the droplets are formed via physical means, the organic solvent leaches out of the droplet into the external aqueous phase before evaporating at the water–air interface. Emulsions are simply created by using a propeller or magnetic bar for mixing the organic and aqueous phases. The organic-phase solvent should be able to dissolve the polymer up to reasonably high concentrations but does not necessarily

need to be a good solvent for the drug. The solvent should be completely or almost completely immiscible in water such that a two-phase system can be easily obtained.

When the drug is not soluble in the organic solvent, it may be encapsulated as a solid provided its form is of small size. Nominally, the size of the drug crystals should be at least an order of magnitude smaller than the desired microparticle diameter in order to avoid large bursts associated with dissolution of larger crystals. Smaller crystals will be more homogeneously distributed throughout the organic droplets created in the emulsion. This results in a solid-in-oil-in-water emulsion (S/O/W) and may be used with any hydrophilic drug.

The most serious challenge with encapsulating hydrophilic materials is loss of drug to the external aqueous phase during the formation of the microparticles. Along with the loss of drug to the external phase, the remaining material may migrate to the surface of the droplet before hardening. To minimize these problems, the organic droplets should be hardened into microparticles as quickly as possible following their formation. The method typically involves the use of a viscous organic solution of polymer and drug and a large secondary volume of water that essentially extracts the organic solvent into the external aqueous phase immediately, thus leaving only the microparticle with encapsulated drug. The highly viscous dispersed phase serves two purposes. First, the volume of volatile organic solvent is at a minimum, facilitating its quick removal from the droplet. Second, highly viscous material will make the migration of the solid drug particles/crystals to the surface of the droplet more difficult, resulting in a more homogenous distribution of drug within the microparticle. As an alternative to S/O/W emulsions, hydrophilic drugs may be encapsulated in a polymer matrix using a multiple water-in-oil-in-water (W/O/W) or oil-in-oil (O/O) emulsions.

Cisplatin, slightly soluble in water (1 mg/mL), has been encapsulated in PLGA microparticles by a number of research groups (239). Particle size ranges have varied between 1 and 300 μm with high encapsulation efficiencies (> 90%). However, depending on the type of emulsion used, a large burst was often observed in the in vitro release profiles. Release times, in vitro, vary between a few days to months depending on the diameter of the microparticles and the molecular weight of the polymer used.

Microparticles prepared from PLGA using a S/O/W emulsion contained 5–15% 5 fluoro-uracile (5-FU) showed high efficiency and drug loadings with a desirable release profile with little to no burst and relatively constant release. Duane T. Birnbaum et all., have prepared 5-FU microparticles from high and low molecular weight PLGAs as well as a mixture of

molecular weights. A S/O/W emulsion was used in conjunction with a highly viscous organic phase and an in-liquid drying process. The resulting microparticles were 50–60 μm in diameter with encapsulation efficiencies as high as 75% and drug loadings as high as 25%. Release profiles in buffered saline from PLGA microparticles showed an initial slow and sustained release with no burst and lasts three or more weeks, depending on the molecular weight of the PLGA samples used, with higher molecular weight polymers yielding formulations with longer controlled-release duration. After the polymer degradation reaches a critical phase, the remaining drug is quickly released over a period of about 1 week. Thus the release is controlled by both diffusion and polymer hydrolysis rates, resulting in a biphasic release profile. The time lag between the slower release phase and the faster release phase can be controlled by using different molecular weight PLGA or a blend of PLGA polymers with differing molecular weights. Because higher molecular weight PLGA will hydrolyze at a slower rate, the initial slow-release phase will last longer when using higher molecular weight PLGA, either alone or in a blend. The release profile can be made monophasic by including low molecular weight PLGA and hydrophilic polyethylene glycol (PEG) in the formulation.

LHRH has been encapsulated in PLGA microparticles using a W/O/W emulsion (240). Microparticles prepared using a W/O/W emulsion containing 75:25 PLAGA (mol wt: 14,000) and 5% LHRH released in vitro for several weeks (>4) with no initial burst of hormone. That a water-soluble drug could be efficiently entrapped in a PLGA microparticle and display no initial burst using the W/O/W method is somewhat unusual and very encouraging for researchers in the field. For example, numerous research groups have used the W/O/W method to encapsulated various proteins (e.g., BSA) and the in vitro release typically displays a moderate to large burst. That LHRH has both high encapsulation efficiency and no initial burst has been attributed to the formation of a micelle-like structure between the PLGA chains and the drug (241). The release of the hormone is then strictly regulated by polymer degradation rather than diffusion.

Hydrophobic drugs are typically much easier to encapsulate because they are often highly soluble in the volatile organic solvents used in the formulations and thus lack the thermodynamic drive to partition to the external aqueous phase. Encapsulation efficiencies greater than 90% are typical, with little manipulation of formulation parameters. Challenges arise only when the solubility of the drug is low in the desired dispersed-phase solvent. In these cases, drug loadings may have to be limited to the maximum concentration obtained in the dispersed phase. Alternatively, it may be possible to use a cosolvent system (e.g.,

dichloromethane and methanol) where the second component is used to increase the concentration of drug in the dispersed phase. Hydrophobic drugs often present less of a challenge to formulate in slowly degradable microparticle systems, relative to hydrophilic drugs, as they are often soluble in the organic solvents used, but are insoluble in water. Many hydrophobicdrugs that have been encapsulated in biodegradable microparticles include (242).

For example taxol has been successively encapsulated in PLGA microparticles with high efficiency (> 90%) (242, 243, 244). The maximum amount of drug encapsulated in the microparticles typically depends on its solubility in the organic solvent used in the formulation. Taxol is now a common anticancer agent used against a wide variety of solid tumors including breast and ovarian cancers. It is insoluble in water and has a limited solubility in ethanol. Taxol has been encapsulated in PLGA microparticles of varying LA/GA ratios using very simple O/W emulsions (244). The authors used dichloromethane as the solvent for both PLGA (mol wt: 10,000) and taxol. A 4% gelatin solution was used as the continuous phase with simple mechanical mixing. An encapsulation efficiency of 98% from microparticles with an average diameter of 30 μm was achieved using 75:25 PLGA with no additional additives. The in vitro release displayed a slow-sustained release of taxol with no initial burst. In fact, the release was so slow that isopropyl myristate was added to change the microparticle matrix to allow the formation of channels that would allow for faster diffusion of taxol from the microparticle. These results are not unexpected considering the hydrophobic nature of the drug and the immiscible nature of the solvents used for both phases of the emulsion. Thermodynamically, taxol must remain solvated in the dichloromethane until which time the solvent is completely removed and, thus, the drug is homogeneously encapsulated in the newly formed microparticle. Because the release of taxol from PLGA microparticles is typically quite slow, the most significant obstacle in formulating these microparticles is obtaining a sustained release of therapeutic levels of drug. Thus, additives such as isopropyl myristate, sucrose, and the use of PLGAs of varying molecular weight and hydrolytic degradation rates have been investigated as a means of accelerating the release of taxol (244, 245).

This biodegradable polymer can be successfully used as controlled delivery systems for many type of drugs and for different route of administration. These systems could provide sustained release of macromolecules ranging from a few days to several months, which avoid the daily multiple administration. Furthermore, encapsulation in polymer matrix also protects labile molecules from the degradation by enzymes. The application of these controlled delivery

systems result in numerous products in the market. These products all base on polyester PLGA due to the favorable regulatory status of the polymer.

3. Aim of the Work

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.

An important consideration in the treatment of pulmonary infections is the fact that the MTB and MAC have the capability of surviving intra-cellulary in the host macrophages for long periods of time (246). Therefore, the ability of the antibacterial agent to eradicate the microrganisms within the macrophage is of key importance.

However, most of the anti-mycobacteria drugs presently in use fail to penetrate macrophages. For this reason, many researchers are considering the use of appropriately engineered delivery systems for these drugs, in order to make them therapeutically effective. It is well known that micro-encapsulation technology can be used to accomplish sustained release of antibiotics, when they are formulated in larger sizes than 50 μm, or to target drug delivery systems to specific cells (i.e., macrophages), when antibiotics are formulated in smaller sizes <10μm.

RFP, one of the first choice drugs for TB and MOTT infections, requires high-doses and prolonged treatment (4–6 months). Moreover, it is known that resistance develops (247), while several side effects have been reported in long term therapy (248). For these reasons, several types of novel drug delivery devices have been proposed and characterized for RFP administration, in order to maximize the therapeutic and minimize the toxic and side effects for this drug (249, 250, 251). Polymeric drug carriers are included between the various types of drug delivery systems proposed. Although experience with synthetic polymers is extensive and encouraging, more recently the trend has been to shift towards natural polymers as alginate and chitosan. Main advantages of these polymers are their low cost and compatibility with the encapsulation of a wide range of drugs, with minimal use of organic solvents. Furthermore, bio-adhesion, stability, safety and approval for human use by the US FDA are additional advantages.

The purpose of the present study was to develop particulate carriers for the delivery of RFP in the treatment of MTB and MAC pulmonary infections, and evaluate their suitability to be delivered to the lungs by nebulization therapy. To this purpose we prepared and characterised RFP-loaded chitosan, PLGA or a mixtures of the two polymers (Chitosan coated PLGA particles) microspheres.

RFP-loaded chitosan microparticles have never been prepared before.

Biodegradable microparticles composed of poly(lactide-co-glycolide) (PLGA) can be considered as a well established drug delivery system, having high potential to serve as carriers for drugs as well as vaccines (252, 253). One such application is the alveolar delivery of RFP. PLGA has been used before for the preparation of RFP loaded microspheres, and several studies have been carried out mainly for evaluation of the formulation parameters that influence the release kinetics of RFP from particles. Furthermore, it has been established that these particles can reach alveolar macrophages after aerosol delivery and enhance the therapeutic effect of RFP *in vivo* (254). In addition, while RFP-loaded PLGA microspheres have been prepared and evaluated *in vivo* previously, no relevant pre-formulation studies involving their behaviour during nebulization, have been carried out.

In the present study we compared chitosan, PLGA or mixtures of the two polymers (Chitosam coated PLGA particles) for the preparation of particulate RFP delivery systems that may be administered to the lungs by nebulization. For this, the three types of particles were prepared and their ability to encapsulate RFP was evaluated. After this, using the same nebulization device we evaluated the nebulization ability of the different types of microparticles prepared.

During this study we also compared properties of freshly prepared and freeze-dried microspheres in order to evaluate freeze-dried formulations for rapid re-hydration just before nebulization.

During this work we studied the influence of several formulation parameters on microparticle size distribution, encapsulation efficiency (E%) and nebulization efficiency (NE%).

Since association of RFP with particles is a prerequisite for achieving high RFP concentrations in alveolar macrophages, the most important characteristic for these particles is their ability to retain the drug during the nebulization process, which was also evaluated. Furthermore, a morphological assessment of the particles by Scanning Electron Microscopy was carried out. Toxicity of the prepared microspheres was also evaluated by using A549 alveolar epithelial cells

4. Rifampicin Loaded Chitosan Microspheres Prepared by Precipitation Method

In this chapter we describe the preparatione and characterization of RFP as a potential effective approach to pulmonary MTB and MAC infection therapy. RFP loaded chitosan microspheres were prepared by using precipitation and precipitation chemical-cross-linking methods. Both of them are simple methods that do not require complex apparatus, special precautions and the use of organic solvents. Additionally, fewer purification steps are necessary than in other described methods. Precipitation chemical cross-linking involves the precipitation of the polymer followed by crosslinking. Precipitation can be done in both cases by sodium sulphate followed by chemical crosslinking using glutaraldehyde (220).

In particular, the influence of several parameters (chitosan concentration, and cross-linking agents) on microparticle size distribution, (E%), nebulization efficiency (NE%) and leakage upon nebulization have been studied. Toxicity of chitosan microspheres were evaluated by using A549 alveolar epithelial cells.

4.1. Materials and Methods

4.1.1. Material

Medium molecular weight chitosan with a deacetilation grade of about 87%, rifampicin (RFP), sodium sulphate, glutaraldehyde (GA) and acetic acid were provide by Sigma Aldrich (Germany). Lyophilised bovine submaxillary glands mucin (type I-S) was purchased from Sigma Aldrich.

A549 cells (Passage 31) was a kind gift from Dr. Ben Forbes (School of Pharmacy, Kings College, London) and were cultured in Ham's F12-K medium Biochrom (Berlin, Germany), supplemented with 10% fetal bovine serum (Gibco BRL Life Technology, Grand Island, NY, USA), 100µg/ml penicillin G (Sigma Aldrich), and 100 µg/ml streptomycin sulphate (Sigma Aldrich) at 37°C in a humidified 95% air and 5% CO_2 environment. Cultures (monolayers in tissue culture flasks, 75 cm^2) were fed with fresh medium every 48 h.

All other reagents were of the highest grade commercially available. Water was always used in demineralised form.

4.1.2. Preparation of RFP-Loaded Chitosan Microspheres

RFP was dissolved in a acqueous solution of acetic acid (2% v/v), chitosan at different concentration were also added. A solution of sodium sulphate (20% w/v) was puted in, drop-wise, during stirring with ultraturrax® at 500 rpm and ultrasonication in a bath-type

ultrasonifier, for 30 minutes. After addition of sodium sulphate, in some formulations, a solution of GA (25% w/w) was also added to evaluate the influence of cross-linking agents.

Microspheres were purified by centrifugation for 15 minutes at 3000 rpm. The obtained sediment then was suspended in water. These two purification steps were repeated twice. All purified particles then were lyophilized.

4.1.3. Characterization of RFP-Loaded Chitosan Microspheres

4.1.3.1. Particle Sizing

Microspheres were analysed for their size and polydispersity index on Nano-ZS (Nanoseries, Malvern Instruments), based on photon correlation spectroscopy, at a scattering angle of 90° and temperature of 25°C. Each measurement was the result of 12 runs. Measurements were carried out for both fresh and freeze-dried samples.

Before counting, the samples were diluted with a 0.05% (w/v) tween 80 water solution in order to prevent precipitation during the measurements. Results are the means of triplicate experiments.

4.1.3.2. Surface Charge (Zeta-Potential)

The surface charge of the microspheres was determined with Nano-ZS (Nanoseries, Malvern Instruments). The measurements were carried out in an aqueous solution of KCl 0,1N. Immediately before the determinations, microspheres were diluted with KCl solution. The measured values were corrected to a standard reference at temperature of 20°. Results are the means of triplicate experiments.

4.1.3.3. Particles Morphology

The Optical Microscopy (OM) (Zeiss Axioplan 2), was used for the determination of the shape of RFP loaded chitosan microspheres. A small drop of microspheres suspension was placed on a clean glass slide. The slide containing RFP loaded chitosan microspheres was mounted on the stage of the microscope and observed.

For scanning electron microscopy (SEM) several drops of the microsphere suspension were placed on an aluminum stub having previously been coated with adhesive. The samples were evaporated at ambient temperature until completely dried, leaving only a thin layer of particles on the stub. All samples were sputter coated with gold-palladium (Polaron 5200, VG Microtech,West Sussex, UK) for 90 seconds (2.2 kV; 20 mA; 150–200A°) under an argon atmosphere. The SEM (Model 6300, JEOL, Peabody, NY) was operated using an acceleration voltage of 10 kV.

4.1.3.4. Measurement of Loading Efficiency of RFP in Chitosan Microspheres

A series of RFP solutions of known concentrations in acetonitrile were prepared, and absorbances were measured in order to generate a standard curve.

The rifampicin content of each lot of microspheres was determined by first extracting the RFP and quantifying the amount of drug spectrophotometrically.

The drug encapsulation efficiency was calculated as the percentage of drug entrapped in microspheres compared with the initial amount of drug recovered in unpurified samples. The concentration of rifampicin contained in each sample was determined by measuring the absorbance on a spectrophotometer at 485 nm.

4.1.3.5 Nebulization Studies of Microspheres

A compressor nebuliser system (Medel Aerofamily, Italy) was used in the study. A volume of 3 ml of sample was used for the nebulization. The aerosols containing RFP-loaded chitosan microspheres were collected in water using a modified 3 stages glass impinger similar to those in Figure 4.1. The impinger device was utilized with the collecting flask containing 3 ml of water to which the aerosol was introduced through a calibrated glass tube and critical orifice delivering the jet of aerosol 5 mm above the bottom of the flask.

After aerosolization (10 minutes), and taking into account the dilution (after measuring the exact volume of dispersion collected), the RFP contained in the impinger were assayed in order to evaluate the effect of nebulization on drug leakage from microspheres and the total amount of formulation nebulised into the apparatus. The Nebulization Efficiency (NE%) of microsphere formulations is defined as the total output of drug collected on the impinger as a percentage of the total drug submitted to nebulization.

$$NE\% = (\text{Aerosolised drug} / \text{Total drug placed in nebuliser}) \times 100$$

Because nebulization can lead to drug leakage, it is important to also determine the nebulization efficiency of the encapsulated drug (NEED%). This parameter is defined as the percentage of aerosolised drug that remains encapsulated after nebulization. A portion of nebulised sample was purified by centrifugation and the amount of drug in the sample before and after centrifugation was assayed.

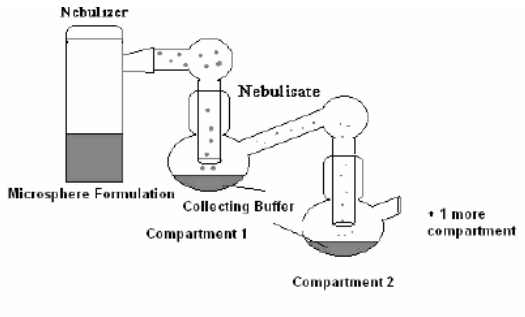


Fig.4.1: Collecting Nebulised Formulations of Microspheres for Evaluating Effects Upon Nebulization

4.1.4. Release/Stability Studies

In vitro release of RFP from chitosan microspheres was determined using, as the release media, phosphate buffer (pH 7.4) and acetate buffer (pH 4.4) in order to simulate the condition in the lungs, and SGF (pH 1.2), in order to evaluate the stability of chitosan microspheres in acidic medium. Freeze-dried formulations were suspended in 500 ml of the dissolution medium, and the amount of microspheres was varied in order to kept constant the amount of drug (25 mg). The experiments were carried out at 37 ± 0.3°C at a rotation speed of 100 ± 2 rpm. A measure of 1 ml samples were withdrawn at appropriate time intervals and centrifuged at 10000 rpm. Supernatants were diluted suitably with acetonitrile and absorbance of the resulting solution was measured at 485 nm in a UV spectrophotometer. The residue (after centrifugation) was redispersed in 1 ml of the fresh dissolution medium and replaced back into the dissolution apparatus. The cumulative amount of RFP was obtained from the calibration curve of RFP in acetonitrile. The stock standard solution of RFP (2 mg/ml) was prepared by dissolving the drug in acetonitrile and storing at 4°C. A standard calibration curve was built up by using standard solutions prepared by dilution of the stock standard solution with acetonitrile.

4.1.5. Mucoadhesive Studies

4.1.5.1. Adsorption of Mucin on Chitosan Microspheres

Bradford colorimetric method (255) was used to determine the free mucin concentration in order to assess the amount of mucin adsorbed on the microspheres and its effect on the assessment of mucoadhesive behavior of chitosan microspheres.

Standard calibration curves were prepared from 2 mL of mucin standard solutions (0.25, 0.5, 0.75, and 1 mg/2 mL). After adding Bradford reagent, the samples were incubated at 37°C for

20 minutes and then, the absorbance of the solution was recorded at 595 nm in a UV spectrophotometer. Triplicate samples were run.

Mucin aqueous solution with different concentrations (0.025, 0.1, and 0.5 mg/mL) were prepared. Freeze-dried chitosan microspheres (20 mg) were dispersed in the above mucin solutions, vortexed, and shaken at room temperature. Then, the dispersions were centrifuged at 4000 rpm for 10 minutes, and the supernatant was used for the measurement of the free mucin content. The mucin content was calculated from the standard calibration curve.

4.1.6. Cell Culture

The human A549 alveolar epithelial cell line (Passage 31) shows similar features as type II alveolar epithelial cells. The cells were grown as monolayers in 35 mm tissue culture dishes incubated in 100% humidity and 5% CO_2 at 37°C. HAM'S medium containing 365 mg/L L-glutamine, supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin was used as the growth media. The cells that form the monolayers were harvested with trypsin (0.25%) centrifuged at low speed (1600 g, 4 min), resuspended in fresh medium and plated at a concentration of 2 x 10^5 cells/dish. The cells were grown to confluence on tissue culture dishes for 3 to 4 days.

4.1.6.1. MTT Assay

For dose-dependent studies, cells were treated with RFP alone and RFP-loaded chitosan microspheres at different concentration in RFP. The effect of RFP in microspheres on the viability of cells was determined by [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] MTT assay (256). The dye is reduced in mitochondria by succinic dehydrogenase to an insoluble violet formazan product. A549 cells (10^5 cells/well) were cultured on 24-well plates with 500 µl of medium for 24 h, with and without the tested compounds. Then 50 µl of MTT (5 mg/ml in PBS) were added to each well and after 2 h, formazan crystals were dissolved in DMSO. Absorbance at 580 nm was measured with a spectrophotometer. On the basis of this assay IC50 values were obtained in three independent experiments for each formulation. In all assays three different concentrations were used. In order to evaluate changes in viability caused by the tested compounds, living cells as well as those in early and late stages of apoptosis and necrosis were counted. All other methods were also carried out after 24 h incubation. The data in this study were expressed as mean ± S.D of at list three experiments.

4.1.7. Statistical Analyses

All experiments were repeated at least three times. Results are expressed as means ± standard deviation. A difference between means was considered significant if the "p" value was less than or equal to 0.05.

4.2. Result and Discussion

4.2.1. Preparation of RFP-Loaded Chitosan Microspheres

The primary goal of this study was to prepare microsphere formulations that could be used to target the delivery of effective doses of RFP to macrophages after aerosol therapy.

Precipitation and precipitation chemical-cross-linking methods are rapid and simple techniques for producing RFP-loaded microspheres with small size and good reproducibility from batch to batch. This production process is based on the solubility behaviour of chitosan, which is poorly soluble in water. Addition of an acid improves its solubility as a result of protonation of amino groups. Chitosan solubility is also affected by other anions present in the solution. In the presence of phosphate, polyphosphate and sulphate ions, chitosan shows a decreased solubility. For this reason, sodium sulphate was chosen for microsphere formulations, since sulphate leads to a poorly soluble chitosan derivative, whereby microsphere formulation become possible.

Composition of RFP-loaded chitosan microspheres is reported in table 4.1.

Table 4.1: Chitosan Microspheres Composition

	RFP	CTS	Sodium Sulphate (20% w/w)	Acetic Acid	GA 25% (w/w)
Form.Ø	2mg/ml	0.20%	0.16 ml	2%	-
Form.1	2mg/ml	0.25%	0.2 ml	2%	-
Form.2	2mg/ml	0.50%	0.4 ml	2%	-
Form.3	2mg/ml	0.75%	0.6 ml	2%	-
Form.Ø G	2mg/ml	0.20%	0.16 ml	2%	+
Form.4	2mg/ml	0.25%	0.2 ml	2%	+
Form.5	2mg/ml	0.50%	0.4 ml	2%	+
Form.6	2mg/ml	0.75%	0.6 ml	2%	+

4.2.2. Size and Morphological Characteristics of Microspheres

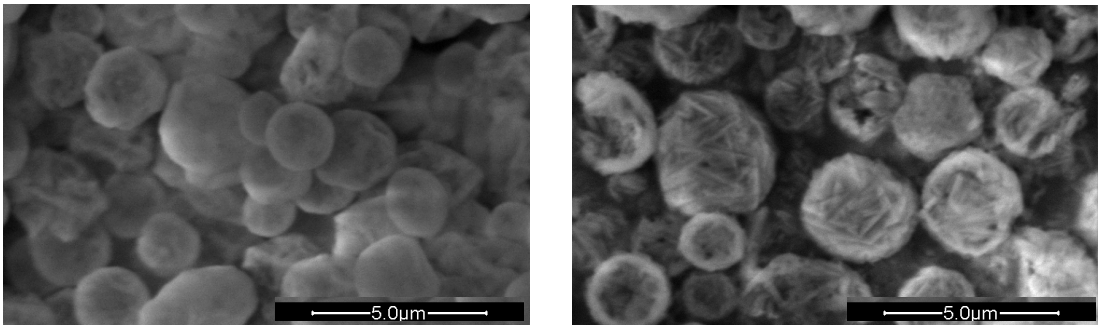
RFP-loaded chitosan microspheres were obtained in the size ranging from 1 to 3 μm. All formulations were monodispersed as shown by the polidispersity index that was always in the range 0.16-0.29 (table 4.2). all particles in this study were also freeze-dried and tha influence of this procedure on microspheres properties was evaluatedin order to design a freeze-dried formulation capable of being rapidly hydrated nad nebulized for the delivery of RFP to lung macrophages. PCS analyses showed that lyophilization did not affect microsphere size (data not shown).

As can be seen from the table, microsphere size increased as chitosan concentration and, therefore, solution viscosity increased. As expected cross-linked microspheres were smaller than the corresponding uncross-linked particles: these differences in size indicate that cross-linked microspheres were more compact in structure because of the cross-linkage.

Table 4.2: Particle Size and Zeta Potential of Chitosan Microspheres

Formulation	Particle Size (nm ± SD)	P.I ± SD	Zeta Potential (mV ± SD)
Form.Ø	NM	NM	NM
Form.1	2310 ± 106	0.160 ± 0.027	+32.5 ± 0.4
Form.2	2470 ± 50.99	0.238 ± 0.011	+34.7 ± 0.1
Form.3	2710 ± 77.88	0.252 ± 0.013	+37.0 ± 0.2
Form.Ø G	NM	NM	NM
Form.4	1470 ± 20.13	0.210 ± 0.089	+23.7 ± 0.6
Form.5	1730 ± 26.30	0.290 ± 0.018	+21.9 ± 0.2
Form.6	2190 ± 47.60	0.243 ± 0.092	+15.6 ± 0.2

Microsphere formation and particle morphology were studied with optical microscopy and SEM. Optical micrographs showed round particles, in a range of size that confirmed PCS measurements. SEM micrographs showed that uncross-linked microspheres were spherical and more regular in shape than the cross-linked ones. As can be seen from figure 4.2, cross-linking with GA gave particles different in shape and with a rough surface.



a **b**

Figure 4.2: Sem Micrographs of Uncross-Linked Particles (a) and Cross-Linked Particles (b)

4.2.3. Surface Charge

Zeta potential has a substantial influence on the stability of suspensions, the interaction of microspheres with charged drugs, and also on the adhesion of drug delivery systems onto biological surfaces. Consequently, investigation of the zeta potential is an important part of microsphere characterization. Phosphate buffer influenced measurement of zeta potential, due to the effect of the counterions on the positively charged chitosan microspheres. For this reason a solution of KCl 0.1N was used for charge surface measurements.

Chitosan microspheres were positively charged (Table 4.2), although sulphate ions were used as precipitant. This indicates that only a part of the amino groups are neutralized during microsphere formation. A different behaviour could be observed between uncross-linked and cross-linked microspheres zeta potential. In fact, while the zeta potential of the uncross-linked particles slightly increased as chitosan concentration increased, the contrary was obtained with the cross-linked particles formulations. Moreover, the zeta potential of these last formulations was smaller than the corresponding uncross-linked formulations 1-3 as a consequence of reduction of amino groups because of interaction with GA.

4.2.4. Entrapment Efficiency (E%)

Figure 4.4 shows the encapsulation efficiency (E%) of the prepared microspheres. In the present work, the influence of chitosan concentration and cross-linking agent on the RFP entrapment in microspheres was evaluated. The highest E% was found in formulations prepared with the lowest amount of chitosan, that are Form. Ø and Form. ØG obtained without and with GA respectively. Chitosan concentration affected considerably the E: in particular the loading capacity increased as the chitosan concentration decreased. This is because the increase in chitosan concentration led to increased solution viscosity that drecreased the loading capacity of the microspheres as a consequence of the reduced drug

solubilization. Part of the drug was not dissolved during preparation process and/or it was probably lose from the microspheres during the washing steps.

GA does not affect significantly the E% that was very similar to that obtained with the corresponding uncross-linked particles.

Encapsulation efficiency were estimated before and after freeze-drying. As can be seen in figure 4.4, freeze-drying does not cause any leakage of the encapsulated drug from the chitosan microspheres. Additionally, it should be stated that the powder produced by the freeze-drying was very easily re-hydrated by one-step addition of the appropriate volume of water, a fact that is important for the desired use intended for the RFP loaded microparticles.

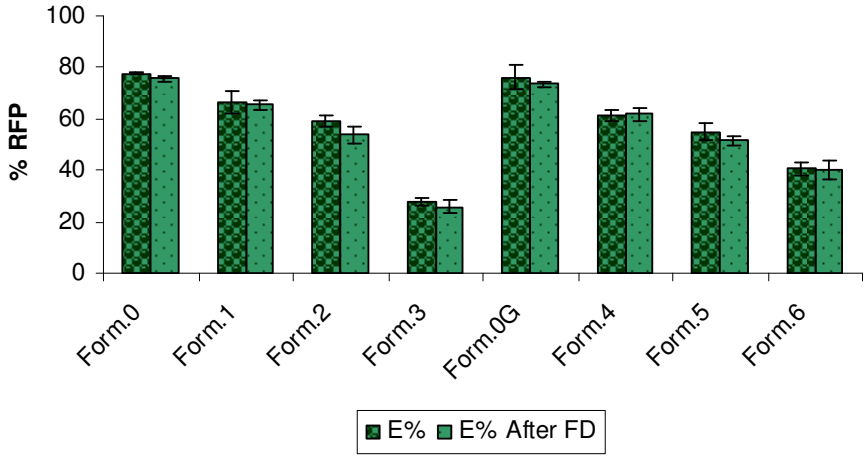


Figure 4.4: Encapsulation Efficiency (E%) Before and After Freeze-Drying

4.2.5. Nebulization Studies of Chitosan Microspheres

Nebulization studies were carried out in order to evaluate the stability and the suitability of chitosan particles for pulmonary/nasal administration. For this reason different analyses were performed on nebulized samples. All these studies were performed by using both freshly prepared and freeze-dried microspheres. It must be pointed that only nebulized particles trapped in water was assayed, while the material deposited on the wall of the impinger was not included in the analysis since it might have dried and disrupted causing drug leakage.

After nebulization, particles size was measured in order to evaluate the effect of this process on mean particle size and size distribution, table 4.3.

Uncross-linked particles, especially Form. 1, showed a decrease in mean particle size. This result could be due to a low stability of the uncross-linked formulations that probably suffered the nebulization energy that caused particle breaking. Cross-linked particles, as can be seen in

the table, did not vary significantly in size after the nebulization process thus confirming their higher stability. All samples were still monodispersed as shown by their low P.I. value.

Table 4.3: Particle Size of Chitosan Microspheres Before and After Nebulization Process

Formulation	Particle Size (nm ± SD) Before Nebulization	P.I ± SD	Particle Size (nm ± SD) After nebulization	P.I ± SD
Form. Ø	NM	NM	NM	NM
Form. 1	2310 ± 106	0.160 ± 0.027	1697 ± 54,62	0,234 ± 0,046
Form. 2	2470 ± 50.99	0.238 ± 0.011	2120 ± 72,11	0,232 ± 0,028
Form. 3	2710 ± 77.88	0.252 ± 0.013	2440 ± 47,44	0,291 ± 0,017
Form. ØG	NM	NM	NM	NM
Form. 4	1470 ± 20.13	0.210 ± 0.089	1770 ± 18,16	0,213 ± 0,076
Form. 5	1730 ± 26.30	0.290 ± 0.018	1693 ± 92,37	0,297 ± 0,022
Form. 6	2190 ± 47.60	0.243 ± 0.092	2480 ± 44,22	0,364 ± 0,054

Using the nebulization device described in the experimental section, we evaluated the nebulization ability of the different types of prepared microparticles. Since association of RFP with particles is a prerequisite for achieving high RFP concentration in alveolar macrophages, the most important characteristic for these particles is their ability to retain the drug during the nebulization process, which was also evaluated.

As can be seen in figure 4.5, the percentage of RFP collected in nebulized microparticle samples (NE%) ranged from 34.35 to 53.0% although the different microparticle formulations showed different nebulization ability for RFP loaded particles. In fact, significant differences were measured for NE% among the different nebulized chitosan microparticles. Indeed, as the chitosan concentration in the particles decreased (which results in a decrease of the particle dispersion viscosity), their NE% increased. A negative effect of viscosity on NE% was demonstrated previously during nebulization of liposomes (257).

However, the cross-linked chitosan particles (with GA) always demonstrated slightly higher NE% compared to the non-cross-linked ones.

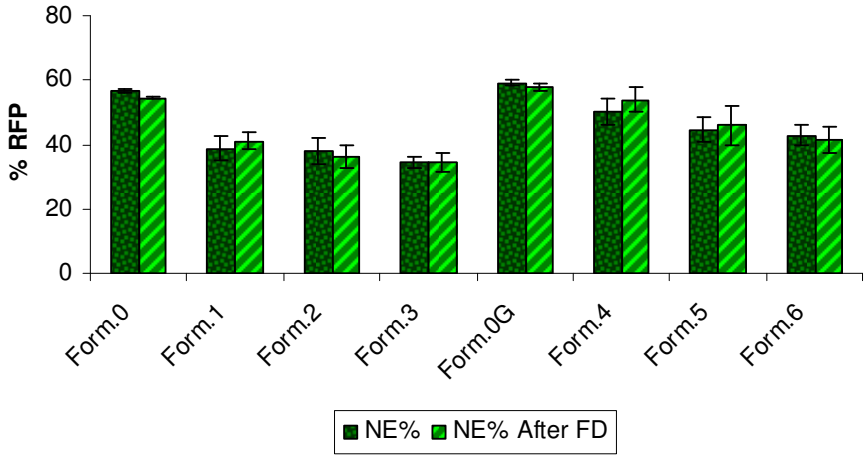


Figure 4.5: Nebulization Efficiency (NE%) Before and After Freeze-Drying

The retention of the drug in the nebulized particles (NEED%) is maybe the most important parameter when a formulation is studied for nebulization delivery. This parameter, in fact, gives important information on the capability of the produced microparticles to retain the encapsulated drug in high amount during the nebulization procedure. Cross-linking chitosan particles with GA definitely resulted in an increased stability during nebulization as it can be concluded by comparing the NEED% values measured for the corresponding uncross-linked chitosan formulations (figure 4.6). In fact, as we said before, GA is able to stabilize chitosan microspheres by immobilizing also the drug encapsulated. NEED% was also affected by the chitosan concentration: it increased as the chitosan concentration decreased.

Freeze-dried microspheres were re-suspended in water and also in this case all the parameters were evaluated. As can be seen in figures 4.5 and 4.6, NE% and NEED% remained almost the same after freeze-drying, and these results suggested that the stability of chitosan microspheres was not affected after liophilization and redispersion process.

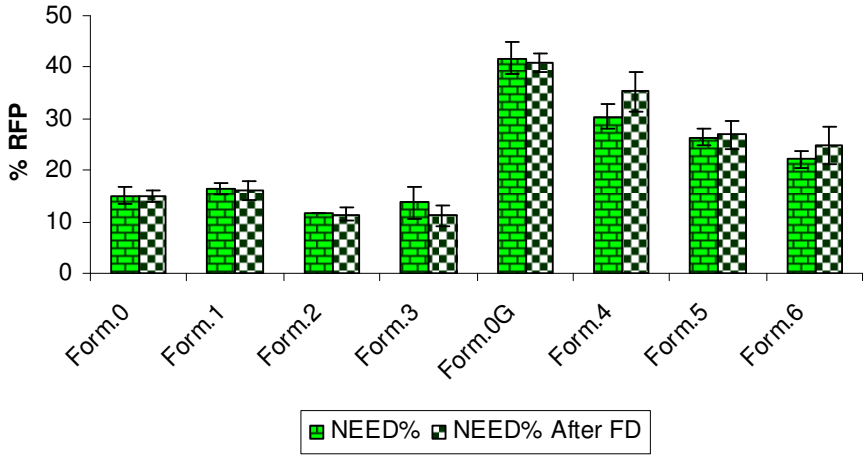


Figure 4.6: Nebulization Efficiency of The Encapsulated Drug (NEED%) Before and After Freeze-Drying

4.2.6. Release/Stability Studies

Release studies were carried out by using three different release media. Phosphate buffer at pH 7.4 and acetic acid buffer at pH 4.4 were used in order to evaluate the influence of the pH inside phagosome and lysosome on RFP release from chitosan microspheres. In Figure 4.7 and 4.8, RFP release profiles from RFP-loaded chitosan microspheres at pH 4.4 and 7.4 buffer solutions respectively, are shown.

As can be seen from the figures, an initial burst effect was observed from all chitosan microparticles (between 19 and 30% of loaded RFP). After this initial burst, all studied microspheres released RFP at a lower rate. RFP release from the was pH dependent (faster release at pH 4.4 than at pH 7,4). This is attributed to the higher solubility of the polymer at lower pH. In fact, as proposed earlier (258), chitosan microspheres can also provide pH-responsive release profile by swelling in acidic environment of the gastric fluid. When comparing the release profiles from cross-linked (with GA) and uncross-linked chitosan microspheres, we see that at pH 7.40 the release of RIF is substantially decreased in the cross-linked particles. It has been proposed before that GA addition in chitosan particles can be used as a method to modulate release kinetics of drugs, as demonstrated for theophylline (259). However, the difference between the release kinetics of RFP from the two types of chitosan particles is more or less diminished (or is a lot smaller) at pH 4.40, possibly due to the rapid swelling and increased solubility of this polymer at low pH, which results in a very fast release of particle- loaded RFP from all chitosan microspheres during the first 8 hours of incubation.

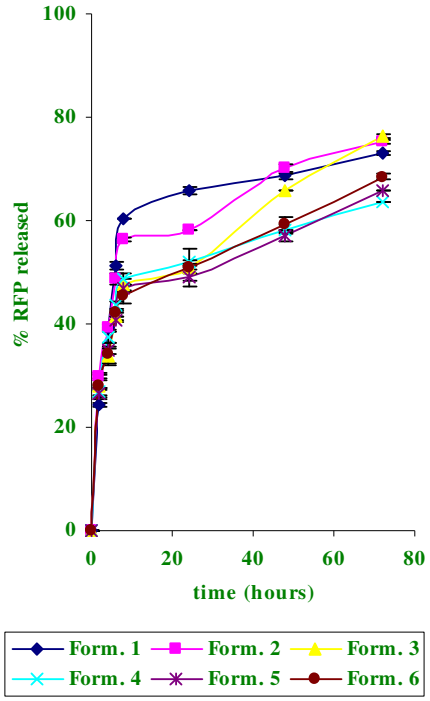


Figure 4.7: Release Studies pH 4.4

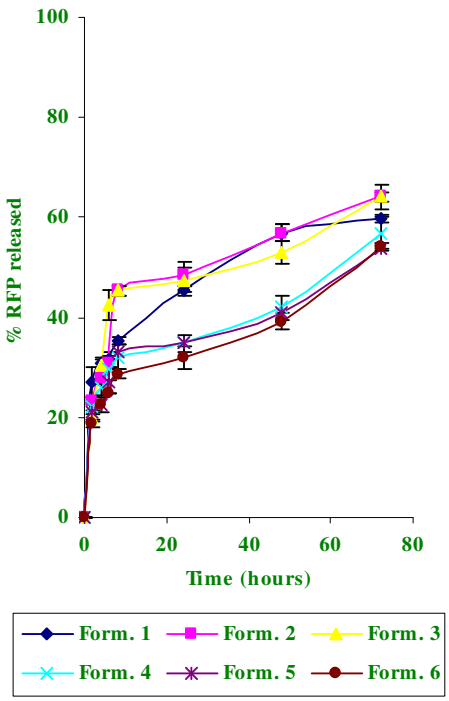


Figure 4.8: Release Studies pH 7.4

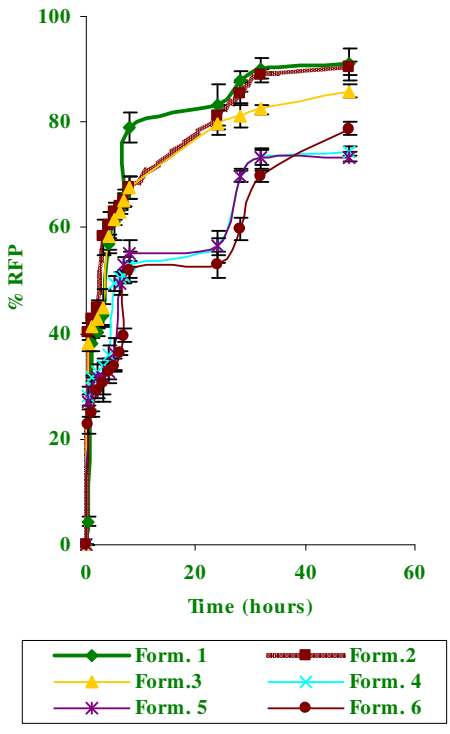


Figure 4.9: Release Studies pH 1.2

Stability of chitosan microspheres at pH 1.2 was also evaluated by studying the drug release. As can be seen in figure 4.9, the release in this acidic medium is even faster than that obtained at pH 4.4. Obviously, this is related to the highly acidic release medium that caused the higher ionization of the D-glucosamine residues with a resulting faster and higher swelling degree, and a very fast release of the drug. As it is evident from the graph, a strong burst effect was found with from 22.3 to 41.5% of RFP released in the first 2 hrs of the experiments. However, also in this case the crosslinking with GA led a decrease of the drug release rate.

4.2.7. Mucoadhesive Studies

Advantages of mucoadhesive properties of particles for inhalation therapy are a cause of argue among scientists. In fact, considering the meaning of mucoadhesion and taking into account the lung mucociliary clearance it could seems a disadvantage. However, it is well known that the mucociliary clearance is more important in the upper airways while particulate adhesion to the mucus layer of the lungs can activate macrophage activity. Finally, it is also important to underline that chitosan exerts a transient inhibitory effect on mucociliary clearance of the bioadhesive formulations due to its surface charge, molecular contact and flexibility, and this is a further advantage for the delivery of RFP to the lungs and in particular to macrophages where the MTB and MAC are able to replicate.

Since a strong interaction exists between mucin and chitosan, mucin should be spontaneously adsorbed to the surface of the chitosan microspheres. For this reason, the mucoadhesive behaviour of chitosan microspheres was assessed by suspending chitosan microspheres in different amounts of mucin aqueous solutions at room temperature. As can be seen in figure 4.10 the amount of mucin adsorbed increased by increasing mucin concentration. These results confirmed that chitosan microspheres have the ability to adsorb mucin. No statistically difference could be observed in the capability to adsorb mucin between uncross- and cross-linked microspheres (Figure 4.10) although a higher mean value of adsorbed mucin was always obtained from the uncross-linked microspheres. The adsorption of mucin to chitosan is expected to be dominated by the electrostatic attraction between positively charged chitosan and negatively charged mucin (the negative charge of mucin is due to the ionization of sialic acid). Therefore, surface charge of chitosan microspheres represented by zeta potential would influenced the amount absorbed. For all the batches the amount of adsorbed mucin decreased with decreasing in zeta potential.

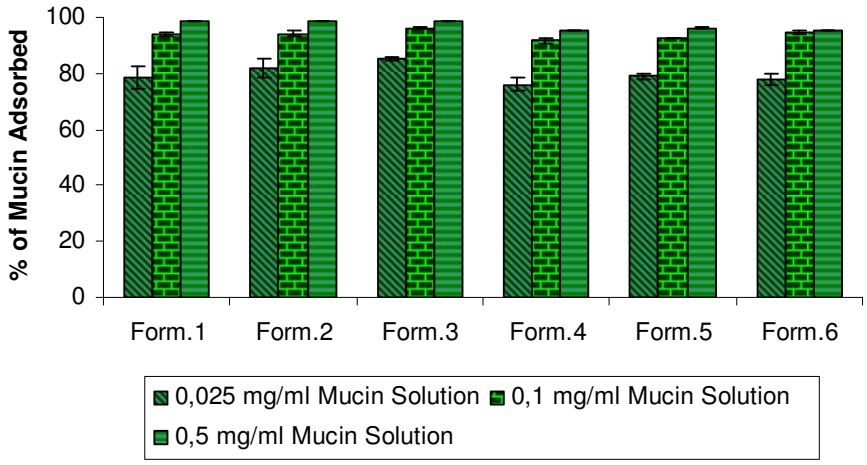


Figure 4.10: Mucoadhesive Studies of Chitosan Microspheres

4.2.8. Viability Studies with A549 Cells

The cytotoxic effects of RFP against A549 cells were examined by MTT assay. In the experiments, the cytotoxicity was evaluated by varying the concentration of free and microsphere-encapsulated RFP. This study was carried out only on Formulation 1 (uncross-linked) and Formulation 4 (cross-linked), which had been prepared with 0.25% of chitosan. Cytotoxicity was observed to be RFP concentration-dependent for all the tested samples (Figure 4.11). The highest cytotoxic effects were found for free RFP, which even in the lowest concentration (0.1%) showed only a 55% cell viability that further decreased to 12% when the free drug was used in the highest concentration (0.5%). These results show the high toxicity of RFP that can cause side effects also when it is nebulized directly into the lung, where is the site of infection. The viability assay also showed that RFP toxicity can be reduced by encapsulating the drug in chitosan microspheres. Cell viability was generally not affected by chitosan concentration, in fact empty particles showed always the best results in term of viability. As can be seen in figure 4.11 the presence of GA decreased the viability of cells probably because same cross-linking agent was in the particles also after the purification steps.

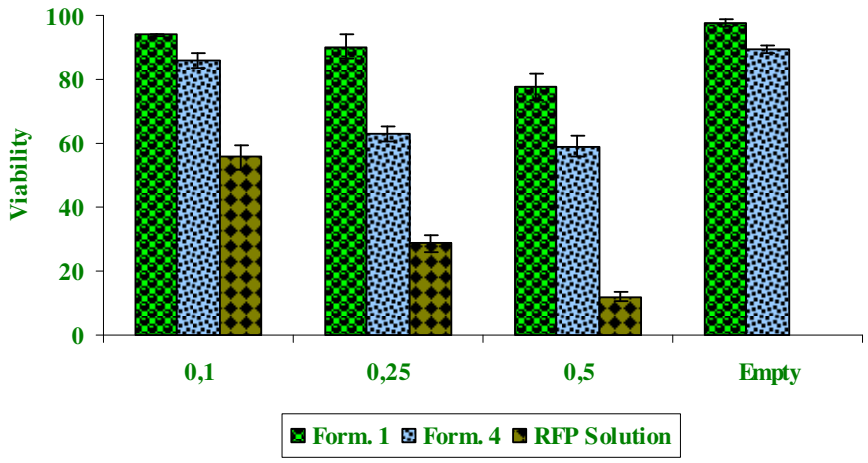


Figure 4.11: Viability Studies whit A549 Cells

4.3. Conclusion

Chitosan is a versatile polymer whose applications range from weight supplement in the market to a drug carrier in formulation research. Cross-linking agents such as GA has been used for preparation of microspheres. The particle size of chitosan microspheres can be modified approximately for the pulmonary delivery of drugs. The entrapment efficiency of drugs in the chitosan microspheres is dependent upon the chitosan concentration. Also the release of drug from chitosan microspheres is dependent upon the concentration of chitosan, but also upon drug content and density of cross linking.

The potential bioadhesiveness of these microspheres are important properties required for the treatment of MTB and MAC infections. Taking together the good bioacceptibility of chitosan, its positive charge that seems to be very advantageous for bioadhesion to the normally negatively charged biological membranes, the suitable release profile for RFP, the possibility to modulate RFP release from chitosan microspheres by adding cross-linking agent (GA), the above presented microspheres may represent useful tools for the delivery of antitubercular drug by aerosol therapy.

5. Rifampicin Loaded Chitosan Microspheres Prepared by Spray-Drying Method

Spray drying is a well-known process, which is used to produce dry powders, granules, or agglomerates from drug-recipient solutions and suspensions. In the present study, RFP entrapped chitosan microspheres, were prepared by using spray-drying method. Particles were characterized for particle size, drug encapsulation efficiency, and in vitro suitability for aerosol delivery.

In particular, preparation and characterization of rifampicin loaded chitosan microspheres as a potential effective approach to pulmonary MTB and MAC infections therapy was studied; the influence of several parameters (chitosan concentration, and cross-linking agents) on microparticle size distribution, entrapment efficiency (E%), nebulization efficiency (NE%) and leakage upon nebulization have been studied.

5.1. Materials and Methods

5.1.1. Material

Medium molecular weight chitosan with a deacetilation grade of about 87%, rifampicin (RFP), GA and acetic acid were provide by Sigma Aldrich Chemie (Germany). Lyophilised bovine submaxillary glands mucin (type I-S) was purchase from Sigma Aldrich.

All other reagents were of the highest grade commercially available. Water was always used in demineralised form.

5.1.2. Preparation of RFP-Loaded Chitosan Microspheres

RFP-loaded chitosan microspheres, obtained by spray drying method, were prepared by dissolving different amounts of chitosan in an aqueous solution of 2% acetic acid (v/v). RFP with and without cross-linking agent (GA 4%, GA) were added to the chitosan solution, under stirring with Ultraturrax® at 500 rpm, and the mixtures were spray-dried from a 0.5 nozzle at a feed rate of 6ml/min (BUCHI Mini Spray Dryer B-290). The inlet and outlet temperature were maintained at 150°C and 95°C, respectively. The spray dried product was collected by a cyclone separator.

Microspheres were purified after redispersion in wather by centrifugation for 15 minutes at 3000 rpm in order to evaluate the encapsulation efficiency. The obtained sediment then was suspended in water. These two purification steps were repeated twice.

5.1.3. Characterization of RFP-Loaded Chitosan Microspheres

5.1.3.1. Particle Sizing and Morphology

The microspheres were analysed for their size and polydispersity index on Dynamic Light Scattering (N4 Plus Beckman Coulter), based on photon correlation spectroscopy, at a scattering angle of 90° and temperature of 25°, using a mathematically constrained regulation (SDP analysis CONTIN program) to overcome the limitation of unimodal analysis for complex distribution. Each measurement was the results of 4 runs.

Before counting, the samples were dissolved with a 0.05% (w/v) tween 80 water solution in order to prevent precipitation during the measurements. Results are the means of triplicate experiments.

5.1.3.2. Surface Charge (Zeta-Potential)

The surface charge of the microspheres was determined with Zetasizer Nano ZS, Malvern Instruments. The measurements were carried out in an aqueous solution of KCl 0,1N. Immediately before the determinations microspheres were diluted with KCl solution. The measured values were corrected to a standard reference at temperature of 20°. Results are the means of triplicate experiments.

5.1.3.3. Particle Morphology

The surface morphology of microparticles was observed by both scanning electron microscopy (SEM) and optical microscopy (OM). The optical microscopy (Zeiss Axioplan 2) was used for the determination of the shape of RFP loaded chitosan microspheres. A small drop of microspheres suspension was placed on a clean glass slide. The slide containing RFP loaded chitosan microspheres was mounted on the stage of the microscope and observed.

The morphological characteristic features of RFP loaded chitosan microspheres were studied using a scanning electron microscope. Spray-dried microspheres were mounted on metal stubs and then coated with a 150Å layer of gold. Photographs were taken using Zeiss-DSM962 Scanning Electron Microscope.

5.1.3.4. Analisi Frattale

5.1.3.5. Measurement of Loading Efficiency of RFP in Chitosan Microspheres

A series of RFP solutions of known concentrations in acetonitril were prepared, and absorbances were measured in order to generate a standard curve. The RFP content of each lot

of microspheres was determined by first extracting the RFP and quantifying the amount of drug spectrophotometrically.

The drug encapsulation efficiency was calculated as the percentage of drug entrapped in microspheres compared with the initial amount of drug recovered in unpurified samples. The concentration of rifampin contained in each sample was determined by measuring the absorbance on a spectrophotometer at 485 nm.

5.1.3.6 Nebulization of Microspheres

RFP-loaded chitosan microspheres aerosols were generated using an efficient high-output continuous-flow Markos Mefar MB2 nebulizer, driven by a Nebula compressor (Markos Mefar) operated at 7l/min. A volume of 3 ml of sample was used for the nebulization. The aerosols containing RFP-loaded chitosan microspheres were collected in a water solution using a modified 3 stages glass impinger. The impinger device was used with the collecting flask containing 3 ml of water to which the aerosol was introduced through a calibrated glass tube and critical orifice delivering the jet of aerosol 5 mm above the bottom of the flask.

After aerosolization (10 minutes), the impinger contents were assayed in order to evaluate the effect of nebulization on drug leakage of microspheres. The secondary aim of this experiment was also to determine the total amount of formulation nebulised into the apparatus. The nebulization efficiency (N.E%) of microsphere formulations is defined as the total output of drug collected on the impinger as a percentage of the total submitted to nebulization.

NE% = (Aerosolised drug /Total drug placed in nebuliser) x 100

Because nebulization can lead to drug leakage, it is important to also determine the nebulization efficiency of the encapsulated drug (N.E.E.D%). This parameter is defined as the percentage of aerosolised drug that remains encapsulated after nebulization. A portion of nebulised sample was purified by centrifugation and the amount of drug in the sample after and before centrifugation was assayed.

5.1.4. Release Studies/Stability Studies

In vitro release of RFP from chitosan microspheres was determined using as the release media, phosphate buffer pH 7.4 in order to simulate some of the conditions in the lungs, and pH 1.2 in order to evaluate the stability of chitosan microspheres in acidic medium. Freeze-dried formulations were suspended in 500 ml of the dissolution medium, and the amount of microspheres was varied in order to kept constant the amount of drug (25 mg). The

experiments were carried out at 37 ± 0.3°C at a rotation speed of 100 ± 2 rpm. A measure of 1 ml samples were withdrawn at appropriate time intervals and centrifuged at 10000 rpm. Supernatants were diluted suitably with acetonitrile and absorbance of the resulting solution was measured at 485 nm. The residue (after centrifugation) was redispersed in 1 ml of the fresh dissolution medium and replaced back into the dissolution apparatus. The cumulative amount of RFP was obtained from the calibration curves of RFP in acetonitrile. The stock standard solution of RFP (2 mg/ml) was prepared by dissolving the drug in acetonitrile and storing at 4°C. A standard calibration curve was built up by using standard solutions prepared by dilution of the stock standard solution with acetonitrile.

5.1.5. Mucoadhesive Studies

5.1.5.1. Adsorption of Mucin on Chitosan Microspheres

Standard calibration curves were prepared from 2 mL of mucin standard solutions (0.25, 0.5, 0.75, and 1 mg/2 mL) and then, the absorbance of the solutions was recorded at 500 nm in a UV spectrophotometer. Triplicate samples were run.

Mucin aqueous solution with different concentrations (0.025, 0.05, 0.1, 0.2, and 0.5 mg/mL) were prepared. Chitosan microspheres (20 mg) prepared by spray-drying method were dispersed in the above mucin solutions, vortexed, and shaken at room temperature. Then, the dispersions were centrifuged at 4000 rpm for 2 minutes, and the supernatant was used for the measurement of the free mucin content. The mucin content was calculated from the standard calibration curve.

5.1.6. Statistical Analyses

All experiments were repeated at least three times. Results are expressed as means ± standard deviation. A difference between means was considered significant if the p value was less than or equal to 0.05.

5.2. Result And Discussion

5.2.1. Preparation of RFP-Loaded Chitosan Microspheres

In order to find the best microsphere formulations for the delivery of effective doses of RFP to macrophages after aerosol therapy, we also prepared and characterized chitosan microspheres of by using an alternative preparation method that is spray drying.

Also in this case uncross- and crosslinked (GA) microparticles were prepared and the influence of different parameters was studied..

Composition of RFP-loaded chitosan microspheres, prepared by the spray drying method, is reported in table 5.1.

Table 5.1: Spray-Dried Chitosan Microspheres Composition

	RFP	CTS	Acetic Acid	GA 4 % (w/w)
Form.A	2mg/ml	0.25%	2%	+
Form.B	2mg/ml	0.50%	2%	+
Form.C	2mg/ml	0.75%	2%	+
Form.D	2mg/ml	1%	2%	+
Form.E	2mg/ml	0.25%	2%	-
Form.F	2mg/ml	0.50%	2%	-
Form.G	2mg/ml	0.75%	2%	-
Form.H	2mg/ml	1%	2%	-

5.2.2. Size and Morphological Characteristics of Microspheres

Spray-dried chitosan microparticles showed a mean particle size ranging from 0.6 to 1.5 μm. As can be seen in table 5.2, microsphere size and polidispersity increased as chitosan concentration and, therefore, solution viscosity increased. This result was probably due to the effect of the solution viscosity on the droplet size during the atomization step. In general, the mean size of droplets formed by atomization is proportional to liquid viscosity and surface tension and it indirectly affects the spray-dried powder size, being an important processing variable.

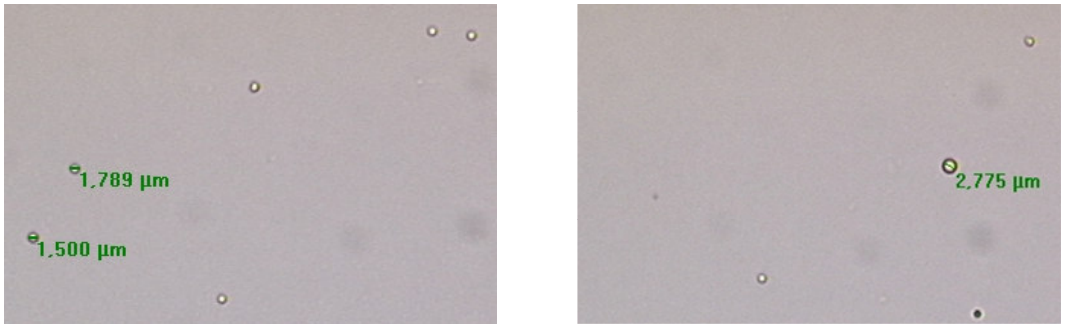
The cross-linking degree controls chitosan microspheres properties and Table 5.2 shows a little increase in the mean size of cross-linked mcirospheres.

Morphology of microparticles was verified with optical microscopy (OM) and SEM. OM images showed round particles, with size in the same range found by PCS analysis. (Figure 5.2a and 5.2b). SEM images showed that microspheres obtained by the spray-drying method were of good morphological characteristics, spherical shape and smooth surface. Figures 5.3a and 5.3b show a sample of both cross-linked and uncross-linked chitosan microspheres (batch A and E respectively). No morphological difference was highlighted for these two different microspheres. Therefore results seem to indicate that GA does not affect the morphological characteristics of cross-linked microspheres obtained by the spray-drying method.

Table 5.2: Particle Size and Zeta Potential of Spry-Dried Chitosan Microspheres

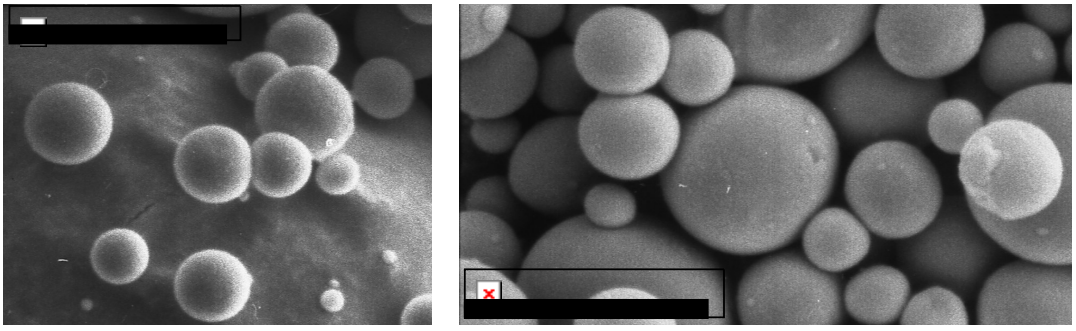
Formulation	Particle Size (nm ± SD)	P.I ± SD	Zeta Potential (mV ± SD)
Form. A	577.0 ± 68.6	0.854 ± 0.020	+23.7 ± 0.6
Form. B	914.3 ± 59.9	0.689 ±0.028	+21.9 ± 0.2
Form. C	1003.2 ± 24.7	0.717 ± 0.020	+15.6 ± 0.2
Form. D	1181.0 ± 121.6	0.593 ± 0.029	+ 15.4 ± 0.1
Form. E	661.5 ± 13.7	0.728 ± 0.030	+32.5 ± 0.4
Form. F	1070.5 ± 103.1	0.215 ±0.027	+34.7 ± 0.1
Form. G	1202.1 ± 27.7	0.840 ± 0.016	+37.0 ± 0.2
Form. H	1306.0 ± 41.1	0.926 ± 0.028	+38.07 ± 0.05

Moreover, SEM micrographs confirmed the high heterogenicity of particle size distribution, which is a consequence of the preparation method. In fact it is well known that using spray-drying method it is not possible to control size distribution of the particles.



a **b**

Figure 5.1: Optical Micrographs of Cross-Linked Spray-Dried Microspheres (a) and Uncross-Linked Spry-Dried Microspheres (b)



a **b**

Figure 5.2: SEM Micrographs of Cross-Linked Spray-Dried Microspheres (a) and Uncross-Linked Spry-Dried Microspheres (b)

5.2.3. Surface Charge

Investigation of zeta potential is an important part of microsphere characterization. This parameter was investigated by using a solution of KCl 0.1N since phosphate buffer could not be used due to the effect of the counterions on the positively charged chitosan microspheres.

All chitosan formulations were positively charged as can be seen in table 5.2.

It was found that the cross-linking with GA led to a decrease of the zeta potential of chitosan microspheres as a consequence of diminution of free amino groups in chitosan structure. In fact, GA reacts with chitosan through the formation of covalent bonds mainly with the amino groups of the polysaccharide.

5.2.4. Entrapment Efficiency (E%)

Figure 5.4 shows the encapsulation efficiency (E%) of the prepared microspheres. All formulations showed very good E%, always ranging between 50% for uncross-linked particles and 80% for cross-linked microspheres. E% of spray-dried microspheres was affected by chitosan concentration: E% decreased as chitosan concentration increased. The increase in chitosan concentration led to highly viscous solutions that reduced drug solubility and therefore the loading capacity.

The influence of the cross-linking agent on the RFP entrapment in chitosan microspheres was also evaluated.

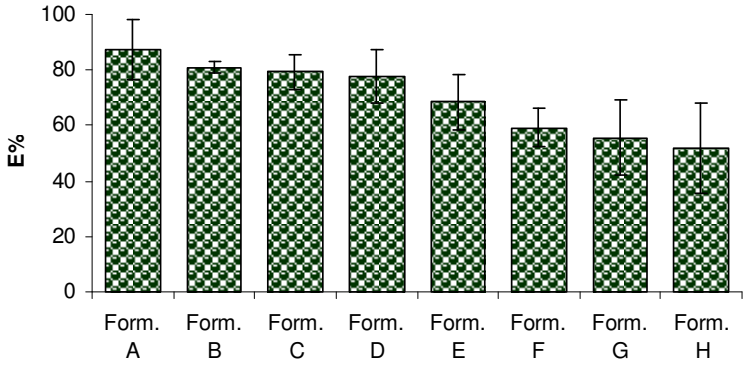


Figure 5.3: Entrapment Efficiency (E%) of Spray-Dried Microspheres

As can be seen in figure 5.4, cross-linked particles (Formulation A-D) showed the best E% because of their stability during atomization and washing steps.

5.2.5. Nebulization Studies of Chitosan Microspheres

Nebulization studies were carried out in vitro in order to evaluate stability and suitability of chitosan microparticles for the pulmonary administration. All these studies were performed by dispersing the spray-dried microspheres in water. RFP-microsphere aerosols, generated using a continuous-flow nebulizer, were collected in a buffer solution in a three stage glass impinger and analysed for the study of nebulization efficiency (NE%) and nebulization efficiency of the encapsulated drug (NEED%) that is the percentage of aerosolized drug that remains encapsulated after nebulization. These studies are of particular importance to have information of the capability of the microspheres to be aerosolized and also to retain the entrapped drug during the process. To this purpose, the study was carried out only on the microspheres that were able to reach the aqueous compartment of the impinger. The microparticles deposited on the impinger walls were not included in the study since their likely dehydration could have caused drug leakage.

During this study, we evaluated the influence of the nebulization process on the particle size. In general, nebulized microspheres showed and increased mean size and a reduced polydispersity index, as can be seen in table 5.3. It is important to underline that formulation H, the most chitosan concentrated uncross-linked formulation, showed the highest increase of particle size. The mean size increase observed in the nebulized particles is probably due to chitosan swelling after their dispersion in water, before the nebulization study. This is particular true for the uncross-linked particles where the swelling process is faster.

Table 5.3:Particle Size of Spray-Dried Microspheres Before and After Nebulization Process

Formulation	Particle Size (nm ± SD) Before nebulization	P.I ± SD	Particle Size (nm ± SD) After nebulization	P.I ± SD
Form. A	577.0 ± 68.6	0.854 ± 0.020	671,3 ± 1,3	0,187 ± 0,013
Form. B	914.3 ± 59.9	0.689 ±0.028	883,4 ± 2,7	0,457 ± 0,030
Form. C	1003.2 ± 24.7	0.717 ± 0.020	1164,1 ± 12,1	0,439 ± 0,041
Form. D	1181.0 ± 121.6	0.593 ± 0.029	1567,5 ± 1,7	0,505 ± 0,021
Form. E	661.5 ± 13.7	0.728 ± 0.030	780,8 ± 13,2	0,155 ± 0,035
Form. F	1070.5 ± 103.1	0.215 ±0.027	1177,2 ± 8,1	0,626 ± 0,022
Form. G	1202.1 ± 27.7	0.840 ± 0.016	1277,8 ± 5,4	0,407 ± 0,012
Form. H	1306.0 ± 41.1	0.926 ± 0.028	2058,3 ± 1,2	0,606 ± 0,040

As can be seen in Figure 5.4, cross-linked formulations A-D showed better NE% than uncrossed linked formulations: almost 80% of rifampicin was recovered after nebulization from formulations A-C. On the contrary, NE% was quite low for the uncross-linked formulations E-H, thus confirming their stability is lower than that of the corresponding GA containing formulations.

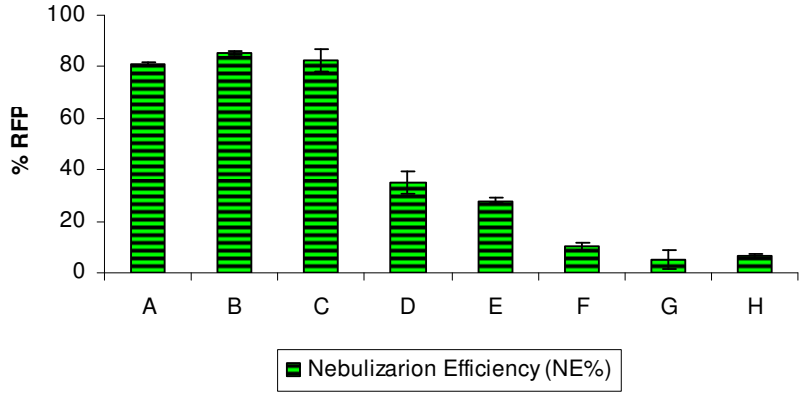


Figure 5.4: Nebulization Efficiency (NE%) of Spray-Dryed Microspheres

Since nebulization can lead to drug leakage, it is also important to evaluate how much of the aerosolized drug is still encapsulated after the nebulization (NEED%). Best results in terms of NEED% were obtained from the cross-linked formulations A-D that were able to retain up to 70% of the encapsulated drug. NEED values were affected by chitosan concentration: they decreased as chitosan concentration increased. Once again, uncross-linked formulations E-H showed very poor nebulization properties since NEED values ranged from 5 to 20%. Results obtained in this analysis seem to point out that the most important factor affecting NEED is polymer cross-linkage, as can be seen by the comparison of NEED values from formulations obtained using the same amount of chitosan in Figure 5.5. Cross-linkage contributes to improve chitosan microsphere stability by immobilizing the encapsulated drug

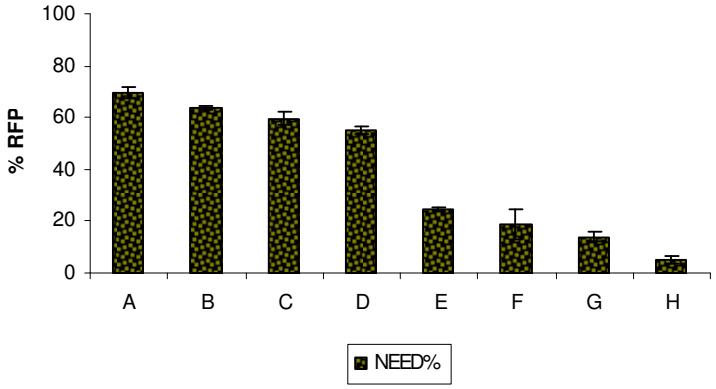


Figure 5.5: Nebulization Efficiency of the Encapsulated Drug (NEED%) of Spray-Dryed Microspheres

5.2.6. Release/Stability Studies

Release studies were carried out by using two different mediums, phosphate buffer at pH 7.4 and SGF at pH 1.2, in order to evaluate the effect of pH on RFP release from chitosan microspheres. In Figure 5.6 and 5.7, RFP release profiles from RFP-loaded chitosan microspheres in both release mediums are shown. Reported values are the arithmetical mean of at list three measurements. Only dissolution profiles from formulations A and E (the most stable and with) the slowest amount of chitosan) are shown in the figure in order to better evaluate the influence of the cross-linking agent, withoth any other variable.

Rifampicin release from the chitosan microspheres is pH dependent: it is faster at pH 1.2 than at pH 7.4. This is the consequence of the higher solubility of chitosan at lower pH, where the D-glucosamine residues are ionized resulting in an extensive polymer swelling and faster drug release. Moreover, rifampicin solubility is pH dependent: it increases as the pH increases. In fact, as proposed earlier, chitosan microspheres can also provide pH-responsive release profile by swelling in acidic environment of the gastric fluid. When comparing the drug release profiles from cross-linked and un-cross-linked chitosan microspheres, a substantial decrease of the release rate is obtained from the cross-linked microparticles at pH 7.4.

As can be seen from the graphs, in both cases there is a significant burst effect, which is more important for formulation E that in only 0.5 hours released 24 (pH 7.4) and 43% (pH 1.2) of the encapsulated drug while formulation A in the same period released 16 and 25% at pH 7.4 and pH 1.2 respectively.

Obtained results show that cross-linkage with GA can delay drug release as a consequence of the higher stability of the hydrogel network. In fact, in both media the cross-linked

formulation A is more capable of controlling the drug release than formulation E that released the 90% of the drug in 48 hours at pH 1.2.

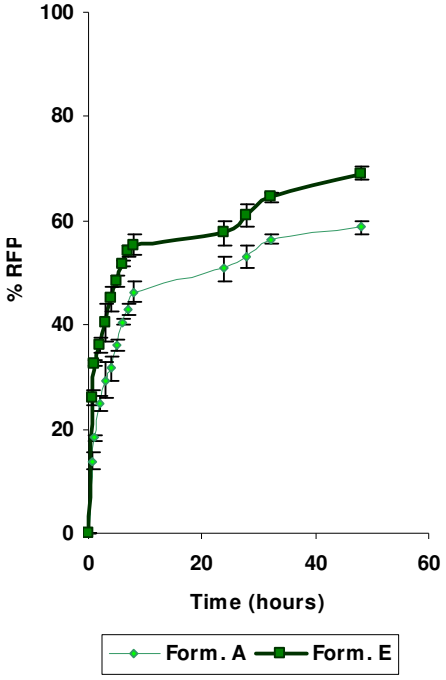


Figure 5.6: Release Studies pH 7.4

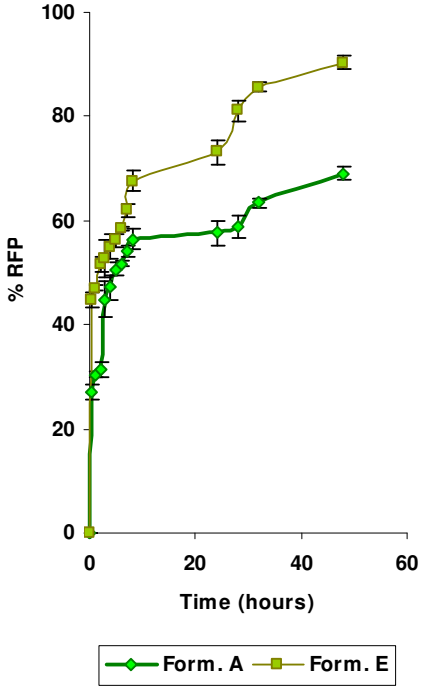


Figure 5.7: Release Studies pH 1.2

5.2.7. Mucoadhesive Studies

Aerosol inhalation therapy via the respiratory tract is desirable for delivering drugs since it has the following advantages over other routes: lung surface area is extremely large and mucosal permeation of drug is comparatively easy because the vascular system is well developed and the walls of alveoli are extremely thin. Finally the intra-cellular or extra-cellular activity of drug-metabolizing enzymes is relatively low.

Since a strong interaction exists between mucin and chitosan, mucin should be spontaneously adsorbed to the surface of the chitosan microspheres. The mucoadhesive behaviour of chitosan microspheres was assessed by their suspension in different amounts of mucin in aqueous solutions at room temperature. As can be seen in Figure 5.8 the amount of adsorbed mucin increased with increasing mucin concentration. These results confirm that chitosan microspheres have the ability to adsorb mucin. The amount of mucin adsorbed was affected by the presence of the cross-linking agent. In fact cross-linked microspheres interacted with a lower amount of mucin, probably because of the cross-linkage and the reduced positive

charge since amino groups are partially neutralized by GA. In fact, for all the batches the amount of mucin adsorbed decreased as the zeta potential of particles decreased. Microspheres with the highest zeta potential (uncross-linked formulations E-H) had the largest amount of adsorbed mucin.

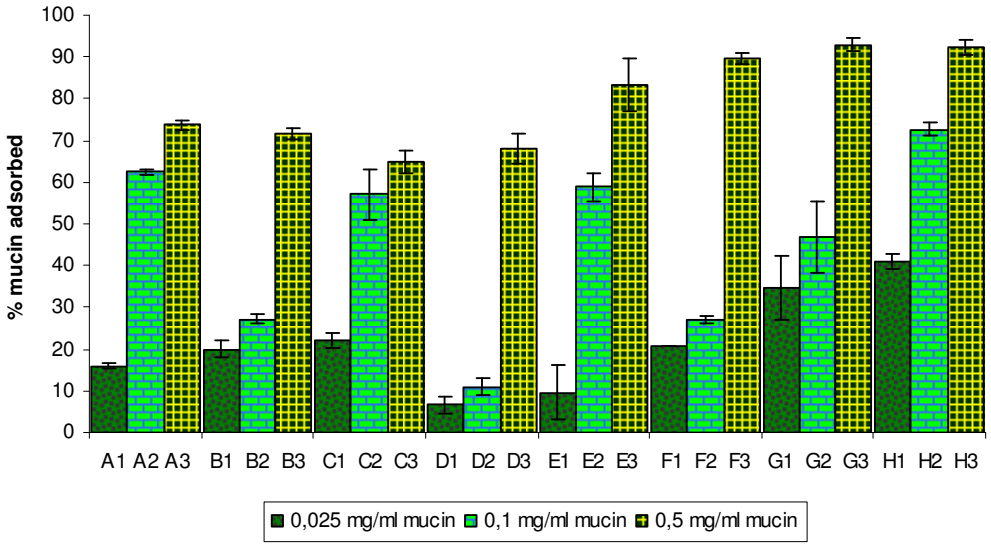


Figure 5.8: Mucoadhesive Studies of Spry-Dried Chitosan Microspheres

5.3. Conclusions

Results obtained during this work showed that the spray drying method is a rapid and simple technique for producing RFP loaded microspheres in good yields, high E% and good reproducibility from batch to batch. In particular, microspheres prepared by this method showed to possess suitable properties for aerosol delivery. In fact comparison of results with those obtained with the precipitation method pointed out that microparticles prepared by the spray-drying process showed higher stability during nebulization.

6. Rifampicin Loaded PLGA Microspheres Prepared by Solvent Evaporation Method

Poly(lactide-co-glycolide) (PLGA) is a the most used polymer owing to its biocompatibility/biodegradability and PLGA microparticles have been successfully employed as ATD-carriers.

PLGA microspheres can be able to achieve slow release rates of RFP in alveolar macrophages that are essential to treat MTB and MAC infections, since these bacillus are facultative intracellular parasites in alveolar macrophages and microspheres can penetrate into the infected macrophages by phagocytosis.

RFP loaded PLGA microspheres were prepared by using solvent evaporation method that need the use of organic solvents ($CHCl_3$).

In this chapter we describe the preparation and characterization of rifampicin loaded PLGA microspheres as a potential effective approach to pulmonary MTB and MAC infections therapy. In particular microparticle size distribution, entrapment efficiency (E%), nebulization efficiency (NE%) and leakage upon nebulization have been studied. The toxicity of PLGA microspheres were evaluated by using A549 alveolar epithelial cells.

6.1. Materials and Methods

6.1.1. Material

PLGA (75:25), rifampicin (RFP), polyvinil alcool (PVA) were provide by Sigma Aldrich Chemie (Germany). Lyophilised bovine submaxillary glands mucin (type I-S) was purchase from Sigma Aldrich.

The A549 epithelial alveolar cell line (passage 31) was a kind gift from Dr. Ben Forbes (School of Pharmacy, Kings College, London) and these cells were cultured in Ham's F12-K medium (Sigma Aldrich), supplemented with 10% fetal bovine serum (Gibco BRL Life Technology, Grand Island, NY, USA), 100 µg/ml penicillin G (Sigma Aldrich), and 100 µg/ml streptomycin sulphate (Sigma Aldrich) at 37°C in a humidified 95% air and 5% CO_2 environment. Cultures (monolayers in tissue culture flasks, 75 cm^2) were fed with fresh medium every 48 h.

All other reagents were of the highest grade commercially available. Water was always used in demineralised form.

6.1.2. Preparation of PLGA Microspheres

PLGA microspheres were prepared by an O/W solvent evaporation method adapted from Prieto et al. (1994). For the preparation of RFP-loaded PLGA microspheres, two different

methods were chosen. In the first PLGA and RFP were dissolved in the organic phase and in the second the drug was dissolved in the aqueous phase. Briefly twenty milligram of PLGA was dissolved in 1 ml of dichloromethane (DCM). This was dispersed in 4 ml of an aqueous phase of 4% PVA. The resultant emulsion was homogenised for 10 min with an Ultraturax® homogeniser at 800 rpm. Subsequent evaporation of the DCM was carried out with mechanical stirring over night at room temperature. Microparticles were collected by centrifugation and washed by dispersion in water with subsequent centrifugation, this step was repeated three times. Microspheres were than freeze dried.

6.1.3. Characterization of RFP-Loaded PLGA Microspheres

6.1.3.1. Particle Sizing and Morphology

The microspheres were analysed for their size and polydispersity index on Zetasizer Nano ZS, Malvern instruments, based on photon correlation spectroscopy, at a scattering angle of 90° and temperature of 25°. Each measurement was the results of 12 run.

Measurements were carried out both for fresh and freeze-dried samples. Before counting, the samples were diluted with a 0.05% (w/v) tween 80 water solution in order to prevent precipitation during the measurements. Results were the means of triplicate experiments.

6.1.3.2. Surface Charge (Zeta-Potential)

The surface charge of the microspheres was determined with Zetasizer Nano ZS, Malvern instruments. The measurements were carried out in an aqueous solution of KCl 0.1N. Immediately before the determinations microspheres were diluted with KCl solution. The measured values were corrected to a standard reference at temperature of 20°. Results are the means of triplicate experiments.

6.1.3.3. Particle Morphology

In preparation for scanning electron microscopy (SEM) several drops of the microsphere suspension were placed on an aluminum stub having previously been coated with adhesive. The samples were evaporated at room temperature until completely dried, leaving only a thin layer of particles on the stub. All samples were sputter coated with gold-palladium (Polaron 5200,VG Microtech,West Sussex, UK) for 90 seconds (2.2 kV; 20 mA; 150–200A°) under an argon atmosphere. The SEM (Model 6300, JEOL, Peabody, NY) was operated using an acceleration voltage of 10 kV.

6.1.3.4. Measurement of Loading Efficiency of RFP in PLGA Microspheres

A series of rifampin solutions of known concentrations in acetonitril were prepared, and absorbances were measured in order to generate a calibration curve. The rifampin content of each lot of microspheres was determined by first extracting the rifampin and quantifying the amount of drug spectrophotometrically.

The drug encapsulation efficiency (E%) was calculated as the percentage of drug entrapped in microspheres compared with the initial amount of drug recovered in unpurified samples. The concentration of rifampin contained in each sample was determined by measuring the absorbance on a spectrophotometer at 485 nm.

6.1.3.5. Nebulization of Microspheres

A compressor nebuliser system (Medel Aerofamily, Italy) was used in this study. 3 ml of purified sample were used for the nebulization studies. The aerosols containing RFP-loaded chitosan microspheres were collected in a water solution using a modified 3 stages glass impinger. The impinger device was used with the collecting flask containing 3 ml of water to which the aerosol was introduced through a calibrated glass tube and critical orifice delivering the jet of aerosol 5mm above the bottom of the flask.

After aerosolization (10 minutes), the impinger contents were assayed in order to evaluate the effect of nebulization on stability of microspheres and the drug leakage during this process. After the experiment was also determined the total amount of formulation nebulised into the apparatus. The nebulization efficiency (N.E%) of microsphere formulations is defined as the total output of drug collected on the impinger as a percentage of the total submitted to nebulization.

NE% = (Aerosolised drug /Total drug placed in nebuliser) x 100

Because nebulization can lead to drug leakage, it is important to also determine the nebulization efficiency of the encapsulated drug (N.E.E.D%). This parameter is defined as the percentage of aerosolised drug that remains encapsulated after nebulization. A portion of nebulised sample was purified by centrifugation and the amount of drug in the sample after and before centrifugation was assayed after complete extraction of the drug from particles with acetonitrile.

6.1.4. Release Studies/Stability Studies

In vitro release of RFP from PLGA microspheres was determined using as the release mediums, phosphate buffer pH 7.4 and acetate buffer at ph 4.0 in order to simulate the

condition in lungs. Freeze-dried formulations were suspended in 500 ml of the dissolution medium, the amount of microspheres was varied in order to kept constant the amount of drug (25 mg). The experiments were carried out at 37 ± 0.3°C at a rotation speed of 100 ± 2 rpm. A measure of 1 ml samples were withdrawn at appropriate time intervals and centrifuged at 10000 rpm. Supernatants were diluted suitably with acetonitrile and absorbance of the resulting solution was measured at 485 nm. The residue (after centrifugation) was redispersed in 1 ml of the fresh dissolution medium and replaced back into the dissolution apparatus. The cumulative amount of RFP was obtained from the calibration curves of RFP in acetonitrile. The stock standard solution of RFP (2 mg/ml) was prepared by dissolving the drug in acetonitrile and storing at 4°C. A standard calibration curve was built up by using standard solutions prepared by dilution of the stock standard solution with acetonitrile.

6.1.5. Mucoadhesive Studies

6.1.5.1. Adsorption of Mucin on PLGA Microspheres

Bradford colorimetric method was used to determine the free mucin concentration in order to assess the amount of mucin adsorbed on the microspheres and its effect on the assessment of mucoadhesive behavior of chitosan microspheres.

Standard calibration curves were prepared from 2 mL of mucin standard solutions (0.1, 0.25, 0.5, 0.75, and 1 mg/2 mL). After adding Bradford reagent, the samples were incubated at 37°C for 20 minutes and then, the absorbance of the solution was recorded at 595nm in a UV spectrophotometer. Triplicate samples were run. All the samples were determined with the same procedure.

The evaluation of mucoadhesive properties was carried out by preparing mucin aqueous solution with different concentrations (0.025, 0.1, and 0.5 mg/mL). PLGA microspheres (20 mg) were dispersed in the above mucin solutions, vortexed, and shaken at room temperature. Then, the dispersions were centrifuged at 4000 rpm for 10 minutes, and the supernatant was used for the measurement of the free mucin content. The mucin content was calculated from the standard calibration curve.

6.1.6. Cell Culture

The human A549 alveolar epithelial cell line shows similar features as type II alveolar epithelial cells. The cells were grown as monolayers in 35 mm tissue culture dishes incubated in 100% humidity and 5% CO2 at 37°C. HAM'S medium containing 365 mg/L L-glutamine, supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100

µg/mL streptomycin was used as the growth media. The cells that form the monolayers were harvested with trypsin (0.25%) centrifuged at low speed (1600 g, 4 min), resuspended in fresh medium and plated at a concentration of 2 x 10^5 cells/dish. The cells were grown to confluence on tissue culture dishes for 3 to 4 days.

6.1.6.1. MTT Assay

For dose-dependent studies, cells were treated with RFP alone and RFP-loaded PLGA microspheres at different concentration in RFP. The effect of RFP in microspheres on the viability of cells was determined by [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] MTT assay (256). The dye is reduced in mitochondria by succinic dehydrogenase to an insoluble violet formazan product. A549 cells (105 cells/well) were cultured on 24-well plates with 500 µl of medium for 24 hours, with and without the tested compounds. Then 50 µl of MTT (5 mg/ml in PBS) were added to each well and after 2 h, formazan crystals were dissolved in DMSO. Absorbance at 580 nm was measured with a spectrophotometer. On the basis of this assay IC50 values were obtained in three independent experiments for each formulation. In all assays three different concentrations were used. In order to evaluate changes in viability caused by the tested compounds, living cells as well as those in early and late stages of apoptosis and necrosis were counted. All other methods were also carried out after 24 h incubation. The data in this study were expressed as mean ± S.D.

6.1.7. Statistical Analyses

All experiments were repeated at least three times. Results are expressed as means ± standard deviation. A difference between means was considered significant if the p value was less than or equal to 0.05.

6.2. Result and Discussion

6.2.1. Preparation of RFP-Loaded PLGA Microspheres

Solvent evaporation method is the most popular technique of preparing PLGA microparticles. It involves emulsifying a drug-containing organic polymer solution into a dispersion medium. Depending on the state of drug in the polymer solution and the dispersion medium, it can be further classified into oil in water (o/w), water in oil (w/o), and water in oil in water (w/o/w) double emulsion method. The o/w method was used in this work. For this technique, drug is dissolved or dispersed in a solution of the polymer in a water-immiscible and volatile organic solvent (DCM). This dispersion is emulsified into an aqueous phase. The organic solvent then

diffuses into the aqueous medium and finally evaporates into the air. In the solvent evaporation method, poly(vinyl alcohol) (PVA) is widely used as an emulsifier in the external aqueous phase and dichloromethane is the most commonly used solvent to dissolve the polymer. The o/w emulsion method was varied in our laboratory in order to make particles by adding the drug not only in the organic phase but also in the aqueous phase.

In fact two types of particles were prepared: the first ones were obtained by dissolving RFP in the organic phase (1) and the second ones were obtained by dissolving RFP in the aqueous phase (2). These different samples were prepared in order to evaluate the influence of drug solubility on different parameters (E%, NE% and NEED%) and on particle characteristics.

Composition of RFP-loaded PLGA microspheres is reported in table 6.1.

Table 6.1: RFP-loaded PLGA Microspheres Composition

	RFP	PLGA	PVA
Form. 1	2 mg/ml	2 mg/ml	4%
Form. 2	2 mg/ml	2 mg/ml	4%

6.2.2. Size and Morphological Properties of PLGA Microspheres

RFP-loaded PLGA microspheres were obtained in the size range around 2 μm and good polidispersity index as shown in table 6.2. The size distribution of PLGA microspheres was evaluated before and after freeze-drying process. Lyophilized microspheres were mixed with water, vortexed for few minutes and than measured. No change in average particle size appeared after lyophilization and resuspension (data not shown).

Table 6.2: Particle Size and Zeta Potential of PLGA Microspheres

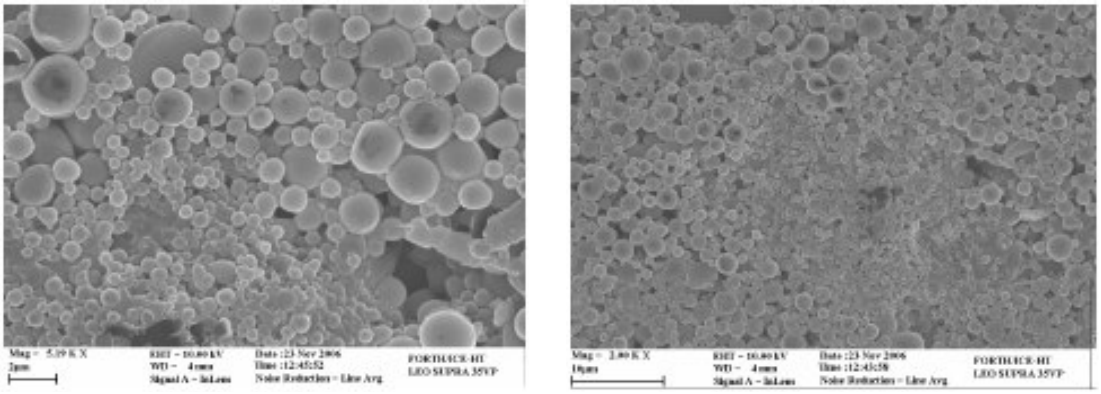
Formulation	Particle Size (nm ± SD)	P.I ± SD	Zeta Potential (mV ± SD)
PLGA 1	2563 ± 49.32	0.192 ± 0.030	-4.60 ± 0.2
PLGA 2	2606 ± 61.10	0.203 ± 0.049	-4.80 ± 0.1

As can be seen in table 6.2 the addition of the drug in the organic or in the aqueous phase did not affect particle size and this is probably because RFP is an amphipatic drug and it can be dissolved in both organic and aqueous phases, although it is more lipophilic.

PLGA microparticles size is normally affected by the presence of an emulsifier. In this case PVA was used to prevent aggregation of the emulsion droplets and polymer sticking during stirring. 4% of PVA was able to stabilize particles also during their storage. This is confirmed by the polydispersity index tha was very low because aggregation did not occur and particles mantained a narrow distribution, as confirmed also by SEM study.

When working with microparticulate systems, it is often helpful to visualize particle shapes and surface characteristics in order to correlate other properties such as surface area and size distribution. RFP-loaded PLGA microspheres prepared using the solvent evaporation method were spherical in shape with a very smooth surface, as shown in Figures 6.1a and 1b. The loading of the antimicrobial agent did not cause any significant change in morphology.

SEM micrographs confirmed the narrow distribution of particles size found by PCS measurement, for each batch. As can be seen, no difference in particle morphology was found as a consequence of the preparation method.



a **b**

Figure 6.1: SEM Micrographs of PLGA Microspheres Obtained by Adding the Drug in the Aqueosus Phase (a) or in the Organic Phase (b).

6.2.3. Surface Charge

Microparticle formulations were characterized also in term of zeta potential because, as well known, it can influence particle stability as well as particle mucoadhesion. In theory, more pronounced zeta potential values, being positive or negative, tend to stabilize particle suspension. The electrostatic repulsion between particles with the same electric charge prevents the aggregation of the spheres. The PLGA particles made by the solvent evaporation method were negatively charged as can be seen in table 6.2 and, hence, were poorly mucoadhesive. At a neutral pH value the mucus layer is an anionic polyelectrolyte. Mucoadhesion is promoted by a positive zeta potential value and, thus, the presence of the

positively charged groups on the particles could lead to electrical charge interactions between the mucus and the particles. PLGA negatively charged microspheres were stable and dit not aggregate as confirmed also by SEM investigation, but this surface property make them poorly mucoadhesive.

6.2.4. Entrapment Efficiency (E%)

Encapsulation efficiency (E%) is an important index to evaluate drug-loaded microspheres as it is more economical when high encapsulation efficiency can be obtained. An O/W emulsion technique is mostly used for the encapsulation of drugs. One objective of this study was therefore to investigate the influence of drug solubility on the RFP entrapment in PLGA microspheres.

Figure 6.2 shows E% of the prepared microspheres. As can be seen, no significant difference was found between formulation 1 and 2. However, formulation 1, wich was obtained by adding the drug in the organic phase, showed a higher E% (72%) than formulation 2 (E% = 65%). This small difference could be related to the higher affinity of RFP for the organic phase.

Encapsulation efficiency was measured before and after freeze-drying in order to evaluate the effect of this process on the drug retention. As can be seen in figure 6.2, freeze-drying did not cause any leakage of the drug encapsulated in chitosan microspheres. It was important to evaluate the effect of the freeze-drying process on this parameter because the aim of this study, like for chitosan particles, was to obtain a stable product, in powder form, able to be rapidly rehydrated just before its use.

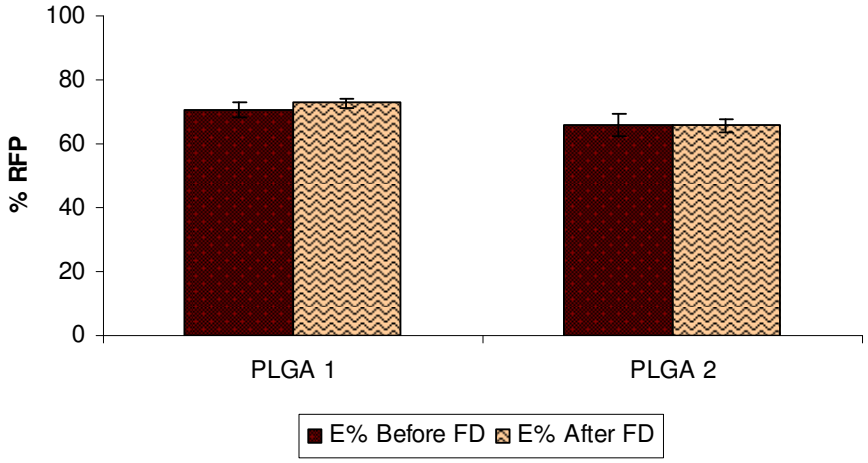


Figure 6.2: Encapsulation Efficiency (E%) of PLGA Microspheres

6.2.5. Nebulization Studies of PLGA Microspheres

Nebulization studies were carried out in order to evaluate the stability and the suitability of PLGA particles for pulmonary/nasal administration. All these studies were performed by using both freshly prepared and freeze-dried microspheres. Different analyses were performed on the nebulized samples. As for chitosan microspheres, only the sample trapped in water was assayed, while the material deposited on the impinger walls was not included.

Size of particles was evaluated after the nebulization process and, as can be seen in table 6.3, there was an important decrease of mean particle size and also a significant increase of polidispersity index. The decrease on mean size during nebulization can be explained taking into account that during this process small particles can be nebulized easily. Nebulization proved that PLGA microspheres, prepared during this work, were multidimensional as it is shown by the high P.I. value. In fact, three different microsphere populations were obtained in the three different impinger stages. The increase in P.I. could also be related to the fact that size analysis was carried out by DLLS, whose sensitivity is not really appropriate for particles in the micron range.

Table 6.3:Particle Size of Spray-Dried Microspheres Before and After Nebulization Process

Formulation	Particle Size (nm ± SD) Before nebulization	P.I ± SD	Particle Size (nm ± SD) After nebulization	P.I ± SD
PLGA 1	2563 ± 49.32	0.192 ± 0.030	1073 ± 35.11	0.778 ± 0.053
PLGA 2	2606 ± 61.10	0.203 ± 0.049	1166 ± 32.61	0.857 ± 0.013

For all microsphere formulations nebulization efficiency and drug leakage after nebulization were evaluated. Both these parameters depend on the stability of particles.

As can be seen in figure 6.3 all formulations showed a good nebulization efficiency (NE%) from 72% for PLGA 1 obtained by adding RFP in the organic phase, to 65% for PLGA 2 obtained by adding RFP in the aqueous phase. During the nebulization process, part of the drug can be lost as a consequence of different processes. In fact, some particles, which are too big, can not be nebulized while others can be disrupted during the process. The little lower NE% of PLGA 2 formulation is probably connected to the preparation method. In fact, by adding RFP in the aqueous phase it is possible to have some drug on the particle surface, from which can be lose easily during the nebulization process.

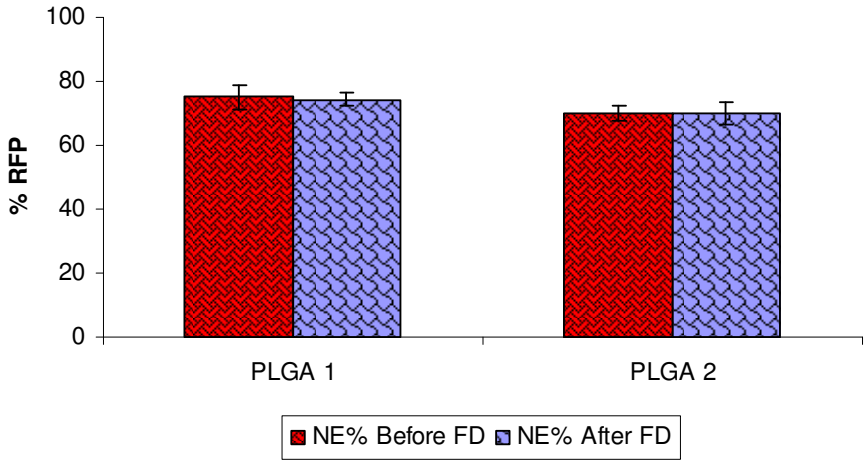


Figure 6.3: Nebulization Efficiency (NE%) of PLGA Microspheres

This behaviour was also confirmed by studing the drug leakage after nebulization. PLGA 1 showed the highest medium NEED% value, (figure 6.4), although no significant difference can be observed between the two formulations. Freeze-dried microspheres were rapidly re-hydrated and also in this case all the parameters (NE% and NEED%) were evaluated. As can be seen in figures 6.3 and 6.4, NE% and NEED% remained almost the same after freeze-drying and these results suggest that the stability of PLGA microspheres was not affected by the liophilization and redispersion process.

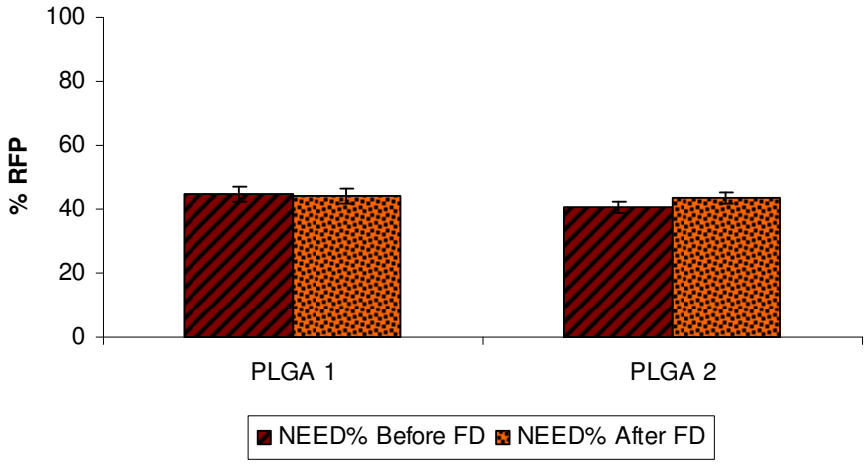


Figure 6.4: Nebulization Efficiency of the Encapsulated Drug (NEED%) of PLGA Microspheres

6.2.6. Release/Stability Studies

In vitro drug release profile reveals fundamental information on the structure (e.g., porosity) and behavior of a formulation on a molecular level, as well as possible interactions between drug and polymer, and their influence on the rate and mechanism of drug release and model release data.

Release studies were carried out by using two different release medium, phosphate buffer at pH 7.4 and acetic acid buffer at pH 4.4, in order to have the same pH values present inside phagosome and lysosome and to evaluate the effect of pH on RFP release from PLGA microspheres. In Figure 6.5 and 6.6, RFP release profiles from PLGA microspheres at pH 7.4 and 4.4 buffer solutions respectively are shown.

The release profiles were very similar in the two different release media. Normally, the release medium pH is able to affect the drug release pattern from PLGA-based microparticles. In this case it was found the same behaviour at pH 7.4 and at pH 4.4 during the 72 hours experiments.

The main reason that can explain this behaviour is correlated to the transition temperature (Tg) of the polymer. In fact it is well know that PLGA polymer Tg is commonly above the physiological temperature of 37 °C, which gives it enough mechanical strength to be fabricated into delivery devices. Tg increases with increase of lactic acid content in copolymer, because the extra methyl group on the lactic acid moiety increases the rigidity of the polymer chain because of the steric hindrance. Moreover, as polymer molecular weight increases a reduced polymer chain mobility is obtained. In fact, increase in the polymer chain length enhances the intra- and interpolymer chain interactions such as chain entanglement and packing, which decrease the polymer chain mobility and consequently increase Tg. On the other hand, it has been found that the release of RFP or other drugs from PLGA particles is influenced by PLGA monomer composition (lactid acid/glycolic acid).

As seen from the results in both cases the PLGA microparticles are able to control RFP release rate, and the initial burst effect demonstrated for this type of particles (12% of RFP loaded in particles) is significantly low if compared to that obtained from chitosan particles. Moreover, after this initial burst, PLGA microspheres released RFP at a lower rate (chapter 4).

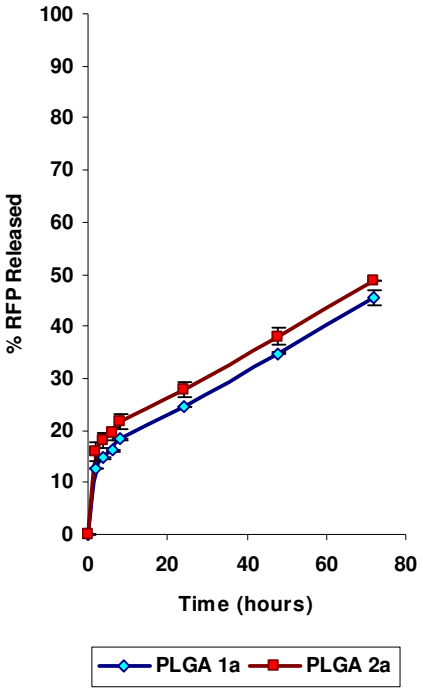


Figure 6.5: Release Studies pH 7.4

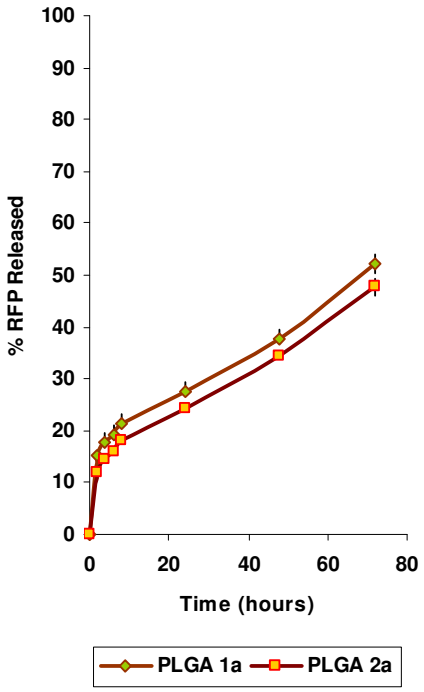


Figure 6.6: Release Studies pH 4.4

6.2.7. Mucoadhesive Studies

The mucoadhesive behaviour of PLGA microspheres was studied at room temperature by suspension of particles in different mucin aqueous solutions at different concentrations. As can be seen in figure 6.7, the amount of mucin adsorbed is very low but it increased with the increasing of mucin concentration. These results confirm that PLGA microspheres are not able to adsorb mucin. This is in agreement with zeta potential data. In fact it is well known that interaction between particles and mucus are prevalently electrostatic attractions but both mucus and PLGA particles are negatively charged and thus they can not interact.

Therefore, the slow amount of mucin adsorbed is only due to the physical entanglement of mucin strands. And the flexible polymer chains (diffusion theory).

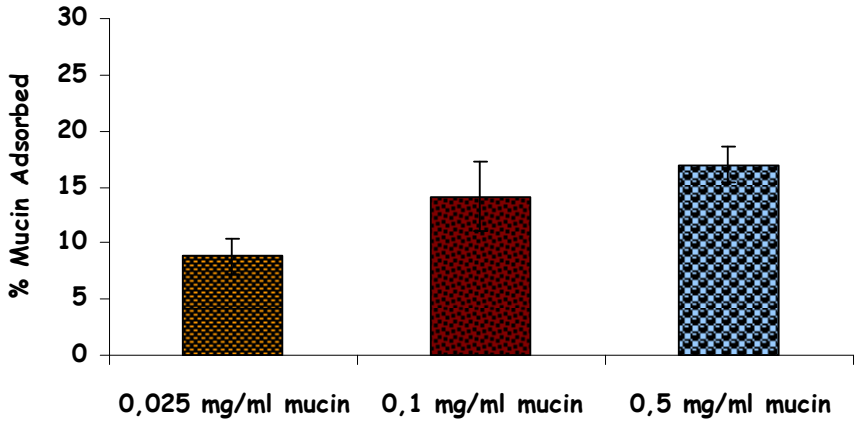


Figure 6.7: Mucoadhesive Properties of PLGA Microspheres

6.2.8. Viability Studies with A549 Cells

The cytotoxic effects of RFP against A549 cells were examined by MTT assay. In the experiments, the cytotoxicity was evaluated by varying the concentration of RFP and RFP entrapped in PLGA microspheres. Cytotoxicity was observed to be concentration-dependent for free RFP and for the formulations. Higher cytotoxic effects were found for free RFP. In fact, as can be seen in figure 6.8, also with the lowest concentration of RFP the viability is very low. This means that the free drug can cause side effects when it is nebulized directly into the lung, where the infection is. Even using the smallest concentration, the free RFP is able to kill the lung cells (epithelial cells similar to A549 cells). As written before, the target of this work was especially the macrophages where mycobacteria are able to survive and replicate. As can be seen from the figure, encapsulation of RFP in the PLGA microspheres led to a decrease of the cytotoxicity, which was quite close to that of the free drug only at the lowest tested RFP concentration .

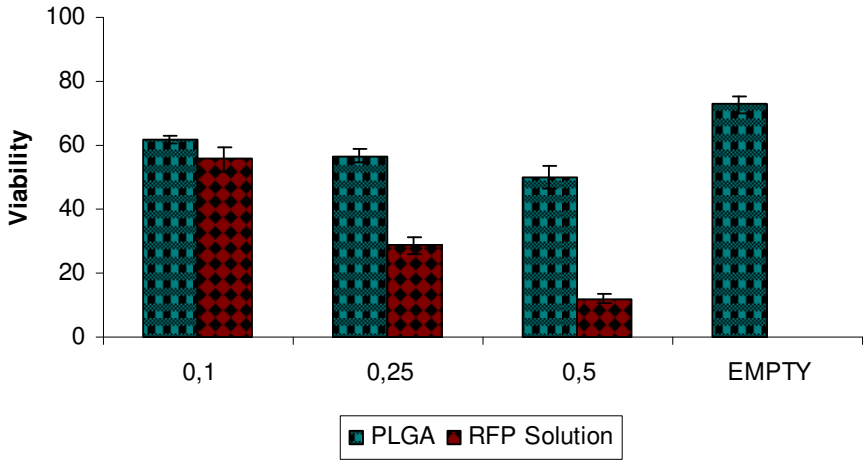


Figure 6.8: Viability Studies with A549 Cells

6.3. Conclusions

Results obtained during this study have shown that PLGA microspheres can lead RFP in very good yields. These particles showed good properties and stability during nebulization.

Summarizing the results of this study, we may conclude that with the exception of their substantially lower mucoadhesive properties, the PLGA polymer is better that chitosan, for the formation of particles that could deliver RFP to alveolar macrophages by nebulisation.

Thereby, an interesting addition to the properties of RIF-loaded PLGA particles would be to improve their mucoadhesive properties through surface modification.

7. Rifampicin Loaded PLGA Coated Chitosan Microspheres Prepared by WSD Method

Biodegradable microparticles made of poly(lactide-co-glycolide) (PLGA) are well-established delivery systems for therapeutics, and have a high potential for peptide, protein and other therapeutic substances. Thanks to its established safety record, PLGA continues to be prevalently used in the field, although the stability of certain entrapped drug like protein, remains a major issue. Nevertheless, one of the typical deficiencies of aliphatic polyesters such as PLGA is their lack of suitable functional groups for an efficient and stable deposition or conjugation of bioactive agents under mild conditions. Surfaces coated with functional polymers or bearing bioactive ligands represent an increasingly desirable feature for drug delivery through microparticulate or nanoparticulate systems besides controlled release and protection of bioactive agents from premature clearance and degradation. Various approaches have been proposed to functionalize the surface of biodegradable microparticles and nanoparticles. The negatively charged surface of PLGA microparticles has been functionalized by the electrostatic binding of cationic surfactants such as cetyltrimethylammonium bromide (260). An alternative to cationic surfactants is the electrostatic coating with polycationic polymers such as chitosan (261, 262, 263). Typically, the establishment of cationic microparticles surfaces may be performed by incubation of previously prepared particles in an aqueous solution of the cationic agent. Direct coating of nascent particles in the course of a typical solvent evaporation/extraction process is achieved by addition of cationic surfactants or polymers to the aqueous extraction phase (264, 265). A third approach to obtain PLGA particles with cationic surface charge is the coencapsulation of a cationic lipid or a polyelectrolyte through spray drying (266). The purpose of PLGA microparticles bearing a cationic surface charge may be to provide a positively charged binding site for the adsorption, condensation and stabilization of nucleic acid biopharmaceuticals (plasmid DNA, mRNA), or antisense oligonucleotides, through electrostatic interaction (267, 268), or to render the particles mucoadhesive, e.g. in view of nasal and pulmonary delivery (269).

Chitosan is highly bioadhesive and has been reported to enhance the permeability of the nasal mucosa (270, 271, 272). Thus, besides offering a substrate for the adsorptive deposition or conjugation of bioactive ligands, chitosan microparticles feature intrinsic bioadhesive and permeation enhancing properties, which make them particularly suitable for nasal and pulmonary administration.

Results obtained with PLGA microspheres led us to develop a novel actively mucoadhesive PLGA microspheres system to improve pulmonary delivery of RFP by coating the particle

surface with the mucoadhesive polymer chitosan. RFP-encapsulated PLGA microspheres coated with chitosan were prepared by the emulsion solvent diffusion method in water (WSD Method).

The influence of chitosan concentration on microparticle size distribution, entrapment efficiency (E%), nebulization efficiency (NE%) and leakage upon nebulization was studied. The toxicity of chitosan microspheres were evaluated by using A549 alveolar epithelial cells.

7.1. Materials and Methods

7.1.1. Material

Medium molecular weight chitosan with a deacetilation grade of about 87% was, PLGA (75:25, mol. Wt 85,2 kDa), rifampicin (RFP), acetic acid, poly-vinyl alcool (PVA) were provide by Sigma Aldrich Chemie (Germany). Lyophilised bovine submaxillary glands mucin (type I-S) was purchase from Sigma Aldrich.

The A549 epithelial alveolar cell line (passage 31) was a kind gift from Dr. Ben Forbes (School of Pharmacy, Kings College, London) and these cells were cultured in Ham's F12-K medium (Sigma Aldrich), supplemented with 10% fetal bovine serum (Gibco BRL Life Technology, Grand Island, NY, USA), 100 μg/ml penicillin G (Sigma Aldrich), and 100 μg/ml streptomycin sulphate (Sigma Aldrich) at 37°C in a humidified 95% air/5% CO2 environment. Cultures (monolayers in tissue culture flasks, 75 cm^2) were fed with fresh medium every 48 h.

All other reagents were of the highest grade commercially available. Water was always used in demineralised form.

7.1.2. Preparation of RFP-Loaded PLGA Coated Chitosan Microspheres

Also in this case WSD Method was used. Chitosan was dissolved in 50 ml of acetic acid buffer solution at pH 4.4, PVA (1%) was also dissolved in this buffer. The PLGA (100 mg) and RFP (2mg/ml) were dissolved in 5 ml of DCM which was poured into 50 ml of aqueous coating polymer solution prepared beforehand at 2 ml/min under stirring at 600 rpm using Ultraturrax® at room temperature. The PVA dissolved in the aqueous solution of coating polymer prevented aggregation of the emulsion droplets and sticking of the polymers to the propeller shaft during agitation. The entire dispersed system was then centrifuged (4500 rpm 15 min) and the sediment was resuspended in distilled water. This process was repeated and the resultant dispersion was then subjected to freeze-drying overnight.

7.1.3. Characterization of RFP-Loaded PLGA Coated Chitosan Microspheres

7.1.3.1. Particle Sizing and Morphology

The microspheres were analysed for their size and polydispersity index on Zetasizer Nano ZS, Malvern instruments, based on Photon Correlation Spectroscopy, at a scattering angle of 90° and temperature of 25°.

Measurements were carried out both for fresh and freeze-dried samples. Before counting, the samples were diluted with a 0.05% (w/v) tween 80 water solution in order to prevent precipitation during the measurements. Results are the means of triplicate experiments.

7.1.3.2. Surface Charge (Zeta-Potential)

The surface charge of the microspheres was determined with Zetasizer Nano ZS, Malvern instruments. The measurements were carried out in an aqueous solution of KCl 0,1N. Immediately before the determinations microspheres were diluted with KCl solution. The measured values were corrected to a standard reference at temperature of 20°. Results are the means of triplicate experiments.

7.1.3.3. Particle Morphology

In preparation for scanning electron microscopy (SEM) several drops of the microsphere suspension were placed on an aluminum stub having previously been coated with adhesive. The samples were evaporated at ambient temperature until completely dried, leaving only a thin layer of particles on the stub. All samples were sputter coated with gold-palladium (Polaron 5200,VG Microtech,West Sussex, UK) for 90 seconds (2.2 kV; 20 mA; 150–200A°) under an argon atmosphere. The SEM (Model 6300, JEOL, Peabody, NY) was operated using an acceleration voltage of 10 kV.

7.1.3.4. Measurement of Loading Efficiency of RFP in PLGA Coated Chitosan Microspheres

The rifampin content of each lot of microspheres was determined by first extracting the rifampin and quantifying the amount of drug spectrophotometrically. A series of rifampin solutions of known concentrations in acetonitril were prepared, and absorbances were measured in order to generate a standard curve.

The drug encapsulation efficiency was calculated as the percentage of drug entrapped in microspheres compared with the initial amount of drug recovered in unpurified samples. The concentration of rifampin contained in each sample was determined by measuring the absorbance on a spectrophotometer at 485 nm.

7.1.3.5. Nebulization of Microspheres

A compressor nebuliser system (Medel Aerofamily, Italy) was used in the study. A volume of 3 ml of sample was used for the nebulization. The aerosols containing RFP-loaded PLGA coated chitosan microspheres were collected in water using a modified 3 stages glass impinger. The impinger device was utilized with the collecting flask containing 3 ml of water to which the aerosol was introduced through a calibrated glass tube and critical orifice delivering the jet of aerosol 5mm above the bottom of the flask.

After aerosolization (10 min.), the impinger contents were assayed in order to evaluate the effect of nebulization on drug leakage of microspheres. It was also important to determine the total amount of formulation nebulised into the apparatus. The nebulization efficiency (N.E%) of microsphere formulations is defined as the total output of drug collected on the impinger as a percentage of the total submitted to nebulization.

NE% = (Aerosolised drug /Total drug placed in nebuliser) x 100

Because nebulization can lead to drug leakage, it is important also to determine the nebulization efficiency of the encapsulated drug (NEED%). This parameter is defined as the percentage of aerosolised drug that remains encapsulated after nebulization. A portion of nebulised sample was purified by centrifugation and the amount of drug in the sample after and before centrifugation was assayed.

After nebulization particle size distribution was measured in order to ervaluate the effect of this process on this parameter.

7.1.4. Release Studies/Stability Studies

In vitro release of RFP from PLGA coated chitosan microspheres was determined using as the release mediums, phosphate buffer pH 7.4 and acetate buffer pH 4.4, in order to simulate the condition in lungs and in particular the conditions inside lysosomes and phagosomes. Freeze-dried formulations were suspended in 500 ml of the dissolution medium, and the amount of microspheres was varied in order to kept constant the amount of drug (25 mg). The experiments were carried out at 37 ± 0.3°C at a rotation speed of 100 ± 2 rpm. A measure of 1 ml samples was withdrawn at appropriate time intervals and centrifuged at 10000 rpm. Supernatants were diluted suitably with acetonitrile and absorbance of the resulting solution was measured at 485 nm. The residue (after centrifugation) was redispersed in 1 ml of the fresh dissolution medium and replaced back into the dissolution apparatus. The cumulative amount of RFP was obtained from the calibration curves of RFP in acetonitrile. The stock

standard solution of RFP (2 mg/ml) was prepared by dissolving the drug in acetonitrile and storing at 4°C. A standard calibration curve was built up by using standard solutions prepared by dilution of the stock standard solution with acetonitrile.

7.1.5. Mucoadhesive Studies

7.1.5.1. Adsorption of Mucin on Chitosan Microspheres

Bradford colorimetric method was used to determine the free mucin concentration in order to assess the amount of mucin adsorbed on the microspheres and its effect on the assessment of mucoadhesive behavior of PLGA coated chitosan microspheres.

Standard calibration curves were prepared from 2 mL of mucin standard solutions (0.25, 0.5, 0.75, and 1 mg/2 mL). After adding Bradford reagent, the samples were incubated at 37°C for 20 minutes and then, the absorbance of the solution was recorded at 595 nm in a UV spectrophotometer. Triplicate samples were run. All the samples were determined with the same procedure. The mucin content was calculated from the standard calibration curve.

Mucin aqueous solution with different concentrations (0.025, 0.1, and 0.5 mg/mL) were prepared. Freeze-dried chitosan coated PLGA microspheres (20 mg) were dispersed in the above mucin solutions, vortexed, and shaken at room temperature. Then, the dispersions were centrifuged at 5000 rpm for 10 minutes, and the supernatant was used for the measurement of the free mucin content.

7.1.6. Cell Culture

The human A549 alveolar epithelial cell line (passage 31) shows similar features as type II alveolar epithelial cells. The cells were grown as monolayers in 35 mm tissue culture dishes incubated in 100% humidity and 5% CO2 at 37°C. HAM'S medium medium containing 365 mg/L L-glutamine, supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin was used as the growth media. The cells that form the monolayers were harvested with trypsin (0.25%) centrifuged at low speed (1600 g, 4 min), resuspended in fresh medium and plated at a concentration of 2 x 105 cells/dish. The cells were grown to confluence on tissue culture dishes for 3 to 4 days.

7.1.6.1. MTT Assay

For dose-dependent studies, cells were treated with both RFP-loaded PLGA coated CTS microspheres at different concentration in RFP. The effect of RFP in microspheres on the viability of cells was determined by [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] MTT assay (256). The dye is reduced in mitochondria by succinic dehydrogenase to

an insoluble violet formazan product. A549 cells (105 cells/well) were cultured on 24-well plates with 500 µl of medium for 24 hours, with and without the tested compounds. Then 50 µl of MTT (5 mg/ml in PBS) were added to each well and after 2 h, formazan crystals were dissolved in DMSO. Absorbance at 580 nm was measured with a spectrophotometer. On the basis of this assay IC50 values were obtained in three independent experiments for each formulation. In all assays three different concentrations were used. In order to evaluate changes in viability caused by the tested compounds, living cells as well as those in early and late stages of apoptosis and necrosis were counted. All other methods were also carried out after 24 h incubation. The data in this study were expressed as mean ± S.D.

7.1.7. Statistical Analyses

All experiments were repeated at least three times. Results are expressed as means ± standard deviation. A difference between means was considered significant if the p value was less than or equal to 0.05.

7.2. Result And Discussion

7.2.1. Preparation of RFP-Loaded Chitosan Microspheres

Microparticulate drug delivery systems are of considerable therapeutic interest. Currently, this field is dominated by the use of poly(lactide-co-glycolide) (PLGA) type microspheres. However, because of the deficiency of suitable functional groups on their surface, conventional PLGA microspheres lack the possibility of surface modifications for specialized targeting or other purposes. Such modifications are thought to improve greatly the effectiveness of microparticulate delivery systems.

To solve this problem, a WSD process to coat conventional PLGA particles by means of a biocompatible polymer, chitosan, was used.

Whit this method a direct coating of nascent particles was achieved in the course of a typical solvent evaporation/extraction process by addition of the polymer to the aqueous extraction phase.

Four chitosan concentrations (0.1%, 0.25%, 0.5% and 0.75%) were chosen in order to evaluate the relationship between the polymer concentration (and the solution viscosity) and different parameters like encapsulation efficiency, stability of microparticles during nebulization process and drug leakage after nebulization.

Composition of chitosan coated RFP-loaded PLGA microspheres is reported in table 7.1.

Table 7.1 : Rfp-Loaded Plga Coated Chitosan Microspheres: Composition

	RFP	PLGA	PVA	Chitosan
No Chit.	2mg/ml	2mg/ml	1%	-
0,1% Chit.	2mg/ml	2mg/ml	1%	0.1%
0,25% Chit.	2mg/ml	2mg/ml	1%	0.25%
0,5% Chit.	2mg/ml	2mg/ml	1%	0.5%
0,75% Chit.	2mg/ml	2mg/ml	1%	0.75%

7.2.2. Size and Morphological Characteristics Of Microspheres

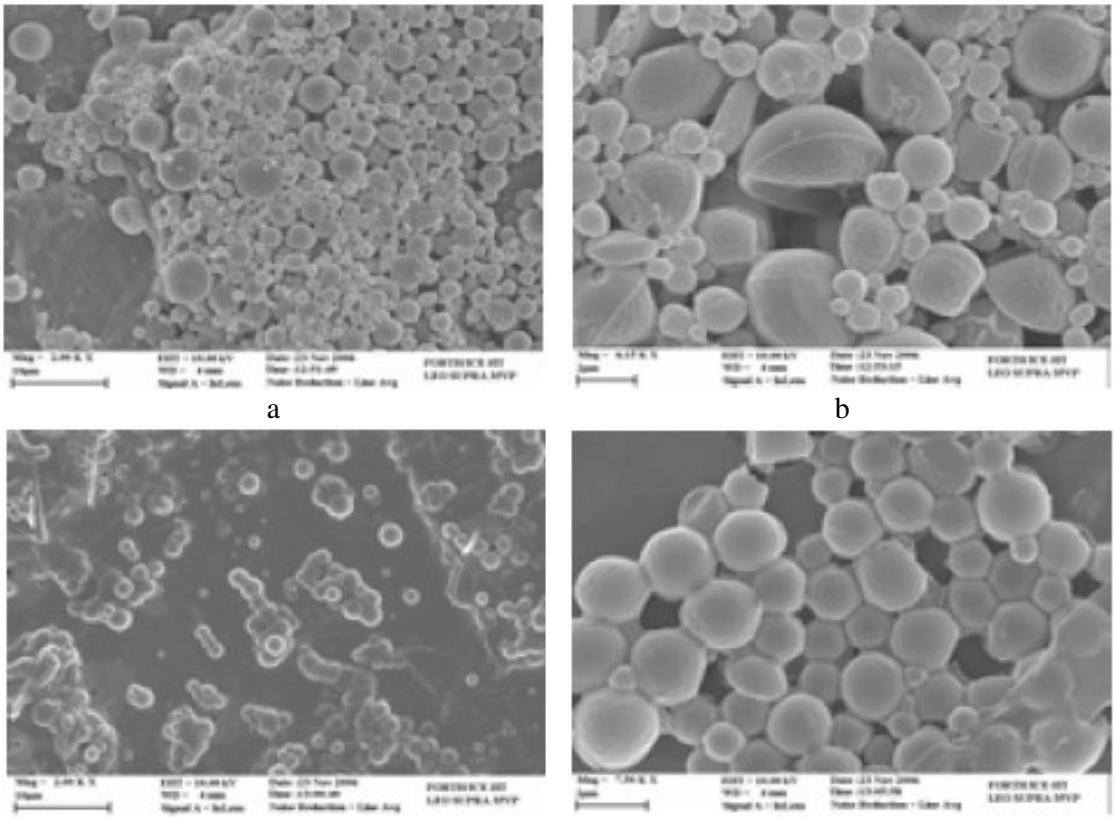
Mean diameter of the surface-modified PLGA microparticles with different amount of chitosan, prepared by the WSD method, are shown in Table 7.2. Average diameter of coated microspheres ranged from 2.9 to 1.5 μm, while uncoated PLGA particles had a mean size of 2,9 μm. Freeze-dried microspheres, readily redispersed in aqueous medium under manual stirring, showed almost the same particle diameter as that before lyophilization, (data not shown).

As can be seen from te table, coating of particles with chitosan led to an increase of microsphere size, which was particularly evident when the lowest amount of the hydrophilic polymer was used. However this is probably due to the stabilizant effect of PVA/chitosan combination and also to increasing interactions between the two polymers (chitosan and PLGA) as the amount of chitosan increased. Moreover, particle-size distribution did not change significantly from batch to batch. In fact, polydispersity index of all prepared batches was always quite big as a consequence of the high polydispersion of the obtained formulations. However, no aggregation could be observed as confirmed by SEM investigations.

Table 7.2: Particle Size and Zeta Potential of RFP-Loaded PLGA Coated Chitosan Microspheres

Formulation	Particle Size (nm ± SD)	P.I ± SD	Zeta Potential (mV ± SD) Withouth RFP	Zeta Potential (mV ± SD) With RFP
No Chit.	2535 ± 58,01	0.506 ± 0,055	+4.70 ± 0.10	-4.80 ± 0.10
0,1% Chit.	2900 ± 59.54	0.571 ± 0.018	+6.90 ± 0.10	+14.9 ± 0.10
0,25% Chit.	2807 ± 85.89	0.459 ± 0.053	+8.40 ± 0.20	+19.10 ± 0.40
0,5% Chit.	2567 ± 19.76	0.494 ± 0.059	+9.40 ± 0.10	+31.10 ± 0.40
0,75% Chit.	1407 ± 51.23	0.392 ± 0.020	+19.50 ± 0.40	+37.00 ± 0.40

As can be seen, a different morphology can be observed from the uncoated and coated particles. In fact SEM micrograph 2a shows that uncoated PLGA microspheres have regular and uniform spherical shape with a smoooth surface. However coating PLGA microparticles with chitosan led to a modification of microspheres surface, which became rough. Figure 7.1.



a b c d

Figure 7.1: Sem Micrographs of RFP-Loaded PLGA Coated Chitosan Microsferes: 0,1% chitosan (a and b), 0,75% chitosan (c and d)

7.2.3. Surface Charge

Zeta potential of microparticles differed significantly between uncoated PLGA and chitosan-coated PLGA microspheres (Table 7.2). Chitosan coating shifted the negative zeta potential of pure PLGA microspheres to positive values, in relation to the used chitosan concentration. Increasing chitosan concentrations shifted the zeta potential from negative to highly positive values, as a consequence of the increasing amount of free ionizable amino groups. But zeta potential is also affected by the presence of RFP. In fact RFP encapsulated microparticles showed an increased zeta potential value. For instance, encapsulation of RFP shifted the zeta potential of uncoated PLGA particles from 2.80mV to -4.80 mV, whereas for chitosan-coated particles the zeta potential values changed from 4mV to 14mV, and from 19mV to 37mV for the lowest chitosan concentration and the highest chitosan concentration respectively (Table 7.2). It means that the zeta potential of the PLGA particles was affected by chitosan concentration in the external phase.

7.2.4. Entrapment Efficiency (E%)

Figure 3 shows encapsulation efficiency (E%) of prepared microspheres. In the present part of the work the influence of chitosan coating concentration on the RFP entrapment in PLGA coated chitosan microspheres was evaluated. The presence of chitosan coating on microspheres enhanced E%, which increased as chitosan concentration increased.

In fact the highest E% were found for formulations with the highest amount of chitosan (0.5% and 0.75%). It has been reported that added hydrophilic polymers to primary emulsion as a stabilizer could prevent loss of drug from the external phase.

The presence of chitosan on particle surface is probably capable of preventing drug leakages during preparation but in particular during purification process (273).

Figure 7.2 also shows that freeze-drying of microparticles did not result in statistically relevant RFP leakage from any of the prepared microspheres.

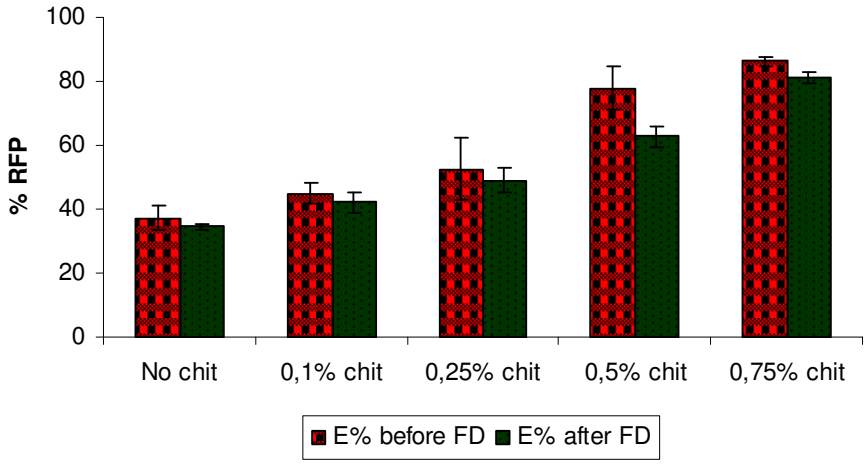


Figure 7.2: Entrapment Efficiency (E%) of Rfp-Loaded Plga Coated Chitosan Microsferes

7.2.5. Nebulization Studies of Chitosan Microspheres

Nebulization studies carried out to evaluate stability and suitability of chitosan coated PLGA particles for pulmonary/nasal administration.

Nebulization led to a reduction of mean particle diameter, but not a reduction of P.I.

These results demonstrate that the prepared microspheres had a multimodal distribution and that nebulization led to a separation of the particles in all the three stages of the used impinger.

Table 7.3: Particle Size of RFP-Loaded PLGA Coated Chitosan Microspheres Before and After Nebulization

Formulation	Particle Size (nm ± SD) Before nebulization	P.I ± SD	Particle Size (nm ± SD) After nebulization	P.I ± SD
No Chit.	2535 ± 58,01	0.506 ± 0,055	1463 ± 50,33	0,637 ± 0,018
0,1% Chit.	2900 ± 59.54	0.571 ± 0.018	1746 ± 25,16	0,558 ± 0,039
0,25% Chit.	2807 ± 85.89	0.459 ± 0.053	1786 ± 38,15	0,523 ± 0,084
0,5% Chit.	2567 ± 19.76	0.494 ± 0.059	1700 ± 32,28	0,754 ± 0,092
0,75% Chit.	1407 ± 51.23	0.392 ± 0.020	1266 ± 32,14	0,756 ± 0,098

For all microsphere formulations nebulization efficiency and drug leakage after nebulization were evaluated. Both of these parameters depend on the stability of particles.

As can be seen in figure 7.3 all coated formulations showed a good nebulization efficiency that increased as chitosan concentration increaed. This is probably because chitosan on the surface can reduce the drug leakage during the nebulization process.

Similarly to chitosan particles described previously (chapter 4 and 5), chitosan coated PLGA microsphere dispersions showed a viscosity that increased as the chitosan concentration increased. However, in this case particles showed good aerodinamic propertiesas they could be easily nebulized (figure 7.4).

A similar trend was observed when NEED% was calculated. As shown in figure 7.5, the amount of RFP still encapsulated after the nebulization was improved by coating the PLGA particles with the hydrophilic polymer.once again, results demonstrated that nebulization properties of the prepared microspheres improved as chitosan concentration increased. In particular formulation 0,75% was able to retain 63,51% of the entrapped drug

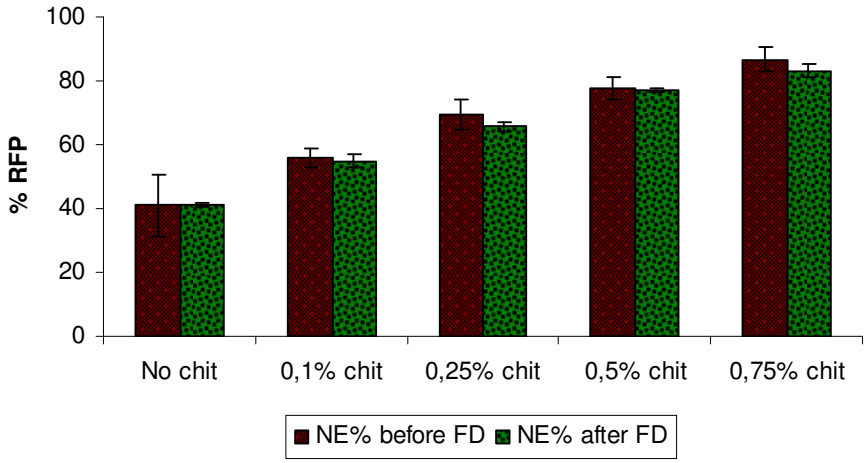


Figure 7.3: Nebulization Efficiency (NE%) of RFP-Loaded Plga Coated Chitosan Microsferes

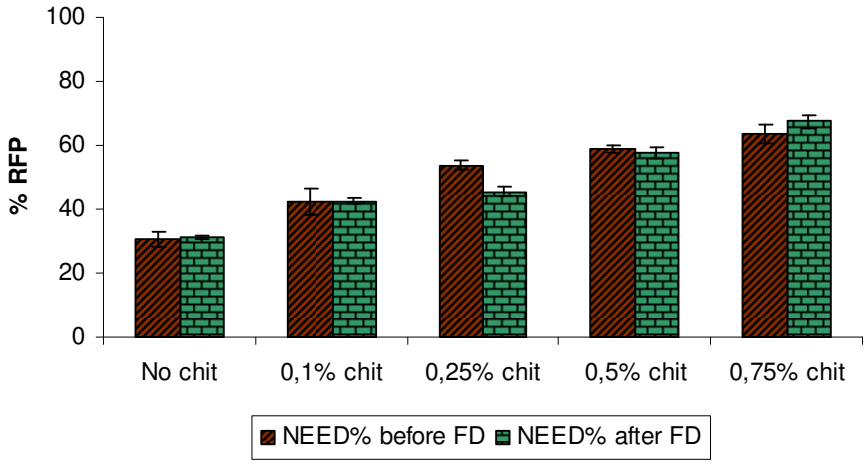


Figure 7.4: Nebulization Efficiency of the Encapsulated Drug (NEED%) of Rfp-Loaded Plga Coated Chitosan Microsferes

Freeze-dried microspheres were re-suspended in water and also in this case all the parameters were evaluated. As can be seen in figures 7.3 and 7.4 NE% and NEED% remained almost the same after freeze-drying and these results suggested that the stability of PLGA coated chitosan microspheres was not affected after liophilization and redispersion process.

7.2.6. Release/Stability Studies

Release studies were carried out by using two different release medium, phosphate buffer at pH 7.4 and acetic acid buffer at pH 4.4, in order to have the same pH values present inside phagosomes and lysosomes and to evaluate the effect of pH on RFP release from chitosan microspheres. In Figures 7.5 and 7.6, RFP release profiles from chitosan coated RFP-loaded PLGA microspheres at pH 7.4 and 4.4 buffer solutions respectively, are shown. At both pH values, about 25% of the drug is immediately released (2 hour) from the uncoated microspheres. This finding indicates that some of the drug is localized on the surface of the microspheres due to the partition of the drug into the surface-active agent layer adsorbed at the surface of the emulsion droplets. After this initial burst, drug release is almost constant, and after 72h 55% of the drug is released from the microspheres. For uncoated PLGA particles the fastest release was observed at pH 7.4 although no difference in the initial burst could be noticed. However, as can be seen drug release from uncoated particles was not affected by the medium pH. On the contrary, coating the microspheres with chitosan altered the drug release pattern: the initial burst was reduced and the reduction increased as chitosan amount increased, from 24 to 7% for uncoated and coated particles respectively.

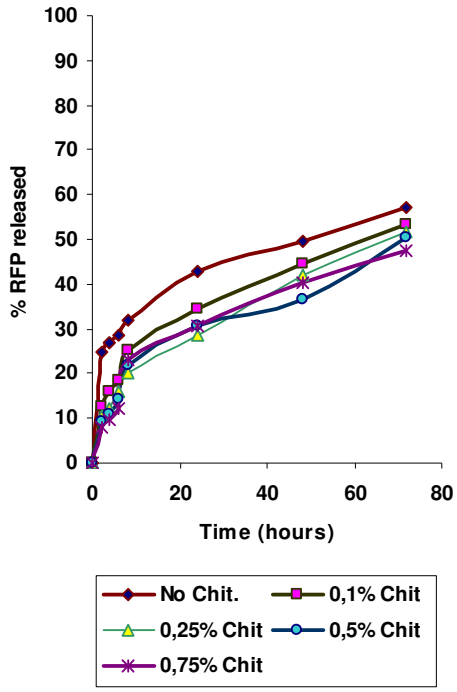


Figure 7.5: Release Studies pH 7.4

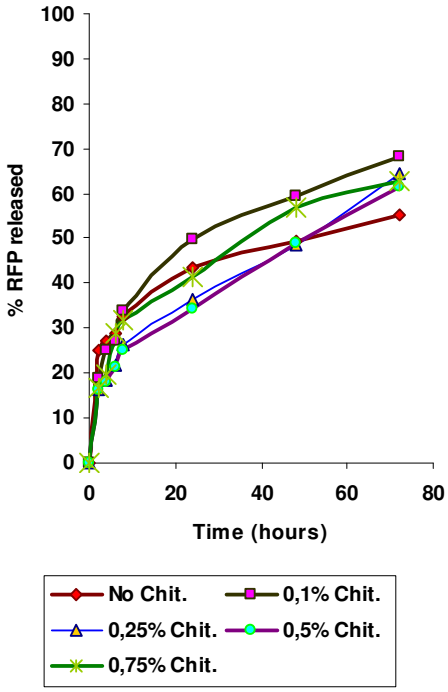


Figure 7.6: Release Studies pH 4.4

This behaviour is more evident at pH 7.4. In fact, at pH 4.4 release from the coated particles is faster even if there is, also in this case, a reduction of the initial burst. Therefore, results confirm the capacity of chitosan on the particle surface to prevent drug leakage in the initial phase of the release. The increased release at pH 4.4 can be explained as a consequence of the ionization of D-glucosamine residues that resulted in extensive swelling and faster drug release.

However, as can be seen in figures 7.5 and 7.6, pH of the release medium does not affect drug release

7.2.7. Mucoadhesive Studies

It has been reported that chitosan has preferable properties for improving drug absorption, such as protection of the drug against enzymatic degradation (274), by opening the intercellular tight junction of the lung epithelium and absorption-enhancing effects in the nasal/pulmonary mucosa (275, 276, 277). However, whether or not chitosan enhances drug absorption in the lungs remains to be determined. For this reason the mucoadhesive properties of RFP-loaded PLGA coated chitosan microspheres were evaluated by measuring the adsorption/association of mucin with the microspheres.

As can be seen from figure 7.7, the chitosan coating of PLGA particles improuves greatly the mucoadhesive properties of the RFP-loaded microspheres. The results confirm those obtained with chitosan microspheres and PLGA particles described above (chapters 4 and 6). Moreover, they are also a logical consequence of the zeta potental values measured (table 7.2). indeed, it is well known that the main mechanism of mucoadhesion is interaction between opposite charged molecules.

Therefore, the positive charge of the chitosan coated microspheres increased the mucoadhesive properties of the negatively charged uncoated PLGA microspheres.

As can be seen from the graph, for chitosan coated particles the percentage of mucin adsorbed increased as the mucin concentration increased from 0.025 to 0.1 mg/ml reaching a 100% mucoadhesion.

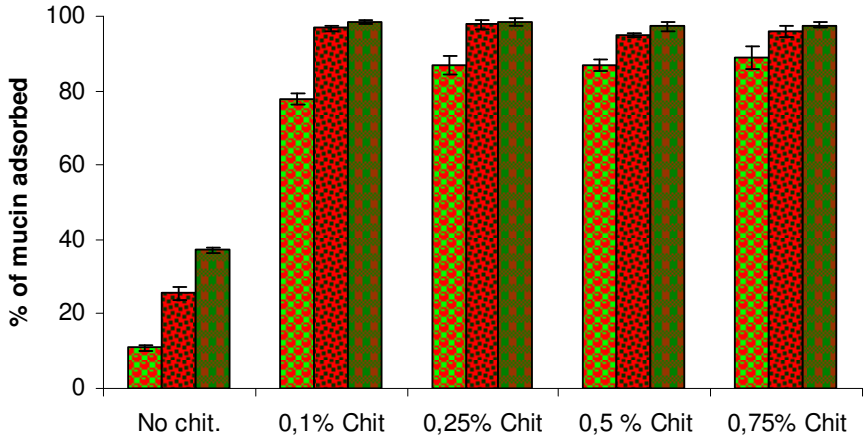


Figure 7.7: Mucoadhesive Behaviour of Rfp-Loaded Plga Coated Chitosan Microsferes

7.2.8. Viability Studies with A549 Cells

The cytotoxic effects of RFP against A549 cells were examined by MTT assay. In the experiments, the cytotoxicity was evaluated by varying the concentration of RFP and RFP entrapped in PLGA coated chitosan microspheres. Cytotoxicity was observed to be concentration-dependent for free RFP and for all the formulations. The highest cytotoxic effects were found for free RFP. In fact, as can be seen in figure 7.8, also with the lowest concentration of RFP the viability is very low and it means that the free drug can cause side effects also when it is nebulized directly into the lung, where the infection is. RFP incorporated in PLGA coated chitosan microspheres were less toxic, but toxicity was still a function of RFP concentration. In fact, empty microspheres were always less toxic than RFP-

coating particles. The highest cellular viability was found for particles more concentrated in chitosan while the lowest for the uncoated PLGA particles. Therefore chitosan reduced toxicity effects of plain PLGA as a consequence of its deposition on the surface of PLGA microspheres.

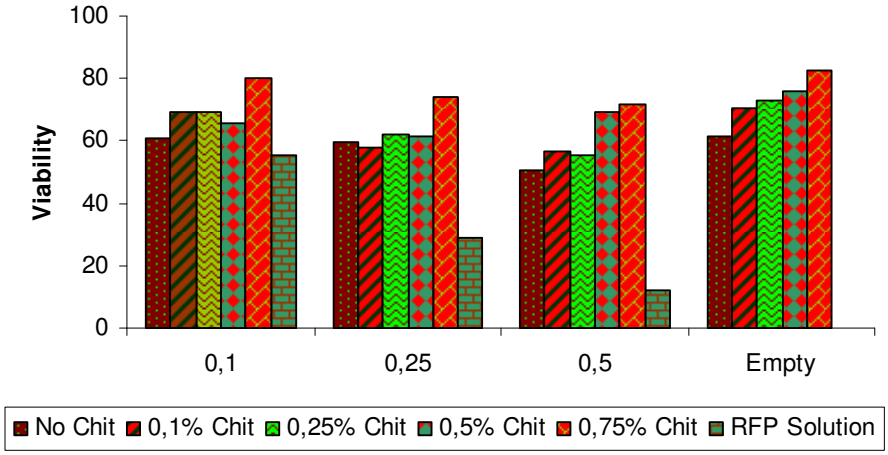


Figure 7.8: Viability Studies of RFP-Loaded PLGA Coated Chitosan Microsferes with A549 Cells

7.3. Conclusion

PLGA microspheres coated with mucoadhesive polymer were successfully prepared by the emulsion solvent diffusion methods in water (WSD). The surface coating of microspheres with chitosan, was confirmed by the change in zeta potential. The excellent mucoadhesion properties of chitosan-coated PLGA microspheres were proved by their significant adsorption to the mucin solution mainly due to the electrostatic attraction between the amino groups of chitosan and the negatively charged mucin. The chitosan-coated PLGA microspheres, which improved the RFP loading, were applied to improve the pulmonary delivery of the drug by nebulization. The particle diameter of the aqueous dispersion of the microspheres was an important factor to enclose the particles in the aerosolized aqueous droplets produced with the nebulizer.

The elimination rate of chitosan-modified microspheres from the lungs can decrease significantly due to the mucoadhesive property after pulmonary administration compared to that of the unmodified microspheres. Furthermore, it was supposed that chitosan on the surface of the microspheres enhances the drug absorption by opening the intercellular tight junctions in the lung epithelium. These findings demonstrated that the chitosan-modified

PLGA microspheres is useful for improving drug delivery via a pulmonary route due to prolonged mucoadhesion for sustained drug release at the absorption site and the absorption-enhancing action of the surface modifier, chitosan.

Viability studies showed that the presence of chitosan on the surface of particles was able to reduce the toxicity of uncoated PLGA particles and also that the increase of chitosan did not affect the viability of A549 cells. However the viability was for both plain RFP and RFP loaded particles, concentration dependent.

8. Final Discussion and Conclusions

In this thesis we described the preparation of RIF-loaded microparticles, designed for use as freeze-dried formulations for rapid re-hydration-nebulization and RFP delivery to lung macrophages. During this study we evaluated particles made with synthetic or natural polymers, (PLGA and chitosan) as well as mixtures of the two polymers (PLGA/chitosan), under identical conditions in order to compare the different cases. Particles were evaluated in terms of RFP loading and stability during freeze-drying and nebulization.

Comparison of the results obtained from the three different particle types, let us to conclude that RFP loading is generally higher in the order PLGA/chitosan > PLGA>chitosan (although this does not apply for all the different samples evaluated in each group). Within the chitosan-group, RFP E% is affected by the chitosan concentration used during chitosan particle formation, but in all cases RFP-loading is considerably lower in chitosan microspheres compared to PLGA. For the PLGA microparticles it seems that RFP loading is higher when RFP is initially added in the system through the organic phase (during microsphere preparation) a fact that is in line with the lipophilic nature of this drug.

Considering the important characteristics of nebulization ability (NE%) and stability (NEED%), the order for particles is again (as for E%) PLGA/chitosan > PLGA > chitosan, while significant differences between the different particle compositions within the groups were observed. For chitosan microparticles NE% increases significantly with decreasing chitosan concentration, due to the easier nebulization of dispersion with lower viscosity (Bridges et al, 2000). The microspheres that exhibited the highest stability during nebulization (PLGA 1 and 2, and PLGA/chitosan 0,75%), were those with a higher compact and stable structure (compared to chitosan particles), as proven by their morphological assessment. The PLGA-based particles showing the highest stability during nebulization were also found to have constant mean diameter before and after the process.

The high stability demonstrated for PLGA-based particles, during nebulization, may be related to the possibility of reaction between the amino groups of RFP and the terminal carboxyl groups of PLGA (278). This also explains the higher E% found for these microparticles than those of chitosan particles. Nevertheless, the fact that chitosan-coated PLGA particles are even better in terms of RFP E%, NE% and NEED% compared to uncoated PLGA particles suggests that chitosan acts as a stabilizer for the particles, possibly by interacting with PLGA (the amino groups of chitosan with the terminal carboxyl groups of PLGA). This explains the almost linear dependence of particle stability on their chitosan content.

Recently, it was proposed that for improved product shelf-life, particles intended for alveolar delivery of drugs could be prepared as freeze dried formulations that will be re-hydrated only prior to nebulization (279). All particles prepared herein were found to be very stable after freeze-drying and the freeze-drying/re-hydration cycle did not affect their NE% and NEED%. Additionally, the quality of the powder produced was always good since it could be easily re-hydrated).

Summarizing the results of this study, we may conclude that the PLGA polymer is better that chitosan, for the formation of particles that could deliver RFP to alveolar macrophages by nebulization. However, the combination of the two polymers at proper quantities results in the formation of very stable microspheres with high loading capacity for RFP.

9. References

1. Fred S. Rosen, The Acquired Immunodeficiency Syndrome (AIDS), Perspectives
2. Friedman-Kie A. E., Laubenstein L. J., Rubenstein P., Bumovici-Klein E., Marmour M., Stahl R., Spigland I., Kim K. S., and Zolla-Paznor S.. Ann. Intern. Med. vol. 96, p. 693-700, 1982.
3. Timothy, Kiehn and Cammarata. Diagnosis of Mycobacterial Infections in Patients with Acquired Immunodeficiency Syndromie, Journal of Clinical Microbiology, vol. 24, p. 708-711, no. 5, 1986.
4. World Health Organization Tuberculosis-Global and regional incidence. Fact sheet N°104March 2006
5. Frieden T.R., Sterling T.R., Munsiff S.S., Watt C.J., Dye C. Tuberculosis (Seminar). The Lancet, vol. 362(9387), p. 887-899, 2003.
6. Skogberg K., Ruutu P., Tukiainen P., Valtonen. Effect of immunosuppressive therapy on the clinical presentation and outcome with tuberculosis. Clin Infect Dis 17: 1012-1017, 1993.
7. Young L.S.. Mycobacterial infections in the immunocompromised host. Curr Op Infect diseases vol. 9, p. 240-245, 1996.
8. Inderlied C.B., Kemper C.A., Bermudez L.E.M.. The Mycobacterium avium complex. Clin. Microbiol. Rev. vol. 6, p. 266-310, 1993.
9. Greene J.B., Sidhu G.S., Lewin S.. Mycobacterium avium-intracellulare: a cause of disseminated life-threatening infection in homosexual and drug abusers. Ann Inter Med vol. 97, p. 539-546, 1982
10. Eng R.H., Bishburg E., Smith S.M., Mangia A.. Diagnosis of Mycobacterium bacteremia in patients with acquired immunodeficiency syndrome by direct examination of blood films. J. Clin. Microbiol. Vol. 27, p. 768-769, 1989.
11. Horsburgh C.R.. Mycobacterium avium complex infection in the acquired immunodeficiency syndrome. New Engl. J. Med. vol. 324, p. 1332-1338, 1991.
12. Spencer P.M., Jackson G.G.. Fungal and mycobacterial infections in patients infected with the human immunodeficiency virus. J. Antimicrob. Chemother. vol. 23 (Suppl. A), p. 107-125, 1989.
13. Horsburgh C.R.. The pathophysiology of disseminated Mycobacterium avium complex disease in AIDS. J. Infect. Dis. vol. 3, p. 461-465, 1999.
14. Chin D.P., Reingold A.L., Stone E.N., Vittinghoff E., Horsburgh C.R., Simon E.M., Yajko D.M., Hadley W.K., Ostroff S.M., Hopewell P.C.. The impact of Mycobacterium avium complex bacteremia and its treatment on survival of AIDS patients: a prospective study. J. Infect. Dis. vol. 170, p. 578- 584, 1994.
15. Bartley P.B., Allworth A.M., Eisen D.P.. Mycobacterium avium complex causing endobronchial disease in AIDS patients after partial immune restoration. Int. J. Tuberculosis Lung Dis. vol. 12, p. 1132-1136, 1999.
16. Morrisey A.B., Aiku T., Falkinham J.O.. Absence of Mycobacterium avium complex disease in patients with AIDS in Uganda. J AIDS vol. 5, p. 477-478, 1992.
17. Horsburgh C.R., Hanson D.L., Jones J.L., Thompson S.E.. Protection from M. avium complex disease in human immunodeficiency virus-infected patients with a history of tuberculosis. J Infect Dis vol. 174, p. 1212-1217, 1996
18. Rosenzweig D. Y.. Pulmonary mycobacterial infections due to Mycobacterium intracellulare-avium complex. Clinical features and course in 100 consecutive cases. Chest vol. 75, p. 115–119, 1979.
19. Wolinski E.. Nontuberculosis mycobacteria and associated diseases. Am. Rev. Respir. Dis. vol. 119, p. 107–159, 1979.
20. Havlik J.A., Horsburgh C.R., Metchock B., Williams P.P., Fann S.A., and Thompson S.E.. Disseminated Mycobacterium avium complex infection: clinical identification and epidemiologic trends. J. Infect. Dis. vol. 165, p. 577–580, 1992.
21. Young L.S. Mycobacterium avium complex infection. J. Infect. Dis. vol. 157, p. 863–867, 1988.

22. Horsbrugh C.R., and Selik R.M. The epidemiology of disseminated nontuberculous mycobacterial infection in the acquired immunodeficiency syndrome (AIDS). Am. Rev. Respir. Dis. vol. 139, p. 4–7, 1989.
23. Walker J.M., and Hannah J.B.. Mycobacterium avium complex infection in patients with the acquired immunodeficiency syndrome. Chest vol. 93, p. 926–932, 1988.
24. Gangadharam P.R..J., and Iseman M.D. Antimycobacterial drugs, In P. K. Peterson and J. Verhoel (ed.), Antimicrobial agents annual 2. Elsevier Science Publishers, Amsterdam, The Netherlands. p. 14–35, 1987.
25. Gangadharam P.R.J., and Iseman M.D. Antimycobacterial drugs, In P. K. Peterson and J. Verhoel (ed.), Antimicrobial agents annual 1. Elsevier Science Publishers, Amsterdam, The Netherlands. p. 17–39, 1986.
26. Benson C.. Mycobacterium tuberculosis and Mycobacterium avium complex disease in patients with HIV infection. Curr Op Infect Dis vol. 7, p. 95-105, 1994.
27. Hughes M.S., Ball N.W., Beck L.A., Lisle G.W., de Skuce R.A., Neill S.D.. Determination of the etiology of presumptive feline leprosy by 16S rRNA gene analysis. Journal of Clinical Microbiology vol. 35, p. 2464-2471, 1997.
28. Greene C.E., Gunn-Moore D.A. Mycobacterial infections. In: Greene CE (ed), Infectious Diseases of the Dog and Cat. Philadelphia: WB Saunders, p. 313-325 1998.
29. Malik R., Wigney D.I., Dawson D., Martin P., Hunt G.B., Love D.N.. Infections on the subcutis and skin of cats with rapidly growing mycobacteria: a review of microbiological and clinical findings. Journal of Feline Medicine and Surgery vol. 2, p. 35-48, 2000.
30. Malik R., Hughes M.S., Love D.N.. Nontuberculous mycobacterial disease. In: August JR (ed), Consultations in Feline Internal Medicine. Philadelphia: WB Saunders, Vol 4, p. 221-232, 2001.
31. Malik R., Hughes M.S., James G., Martin P., Wigney D.I., Canfield P.J., Chen S.C.A., Mitchell D.H., Love D.N. Feline leprosy: two different clinical syndromes. Journal of Feline Medicine and Surgery vol.4, p. 43-59, 2002.
32. Hughes M.S., James G., Ball N., Scally M., Malik R., Wigney D.I., Martin P., Chen S., Mitchell D., Love D.N. Identification by 16S rRNA gene analyses of a potential novel mycobacterial species as an etiological agent of canine leproid granuloma syndrome. Journal of Clinical Microbiology vol. 38, p. 953-959, 2000.
33. Hughes M.S., James G., Taylor M.J., McCarroll J., Neill S.D., Chen S.C.A., Mitchell D.H., Love D.N., Malik R. PCR studies of feline leprosy cases. Journal of Feline Medicine and Surgery vol. 6, p. 235-243. 2004.
34. Appleyard G.D., Clark E.G. Histologic and genotypic characterization of a novel Mycobacterium species found in three cats. Journal of Clinical Microbiology vol.40, p. 2425-2430, 2002
35. Matthews J.A., Liggitt H.D.. Disseminated mycobacteriosis in a cat. Journal of the American Veterinary Medical Association. vol. 183, p. 701-702, 1983
36. Collins F. Acquired resistance to mycobacterial infection. Adv Tuberc Res. vol. 18, p. 1-30, 1972.
37. Ryan K.J.; Ray C.G. Sherris Medical Microbiology, 4th ed., McGraw Hill. ISBN 0838585299. 2004.
38. Cole S.T., Brosch R., Parkhill J., Garnier T., Churcher C., Harris D., Gordon S.V., Eiglmeier K., Gas S., Barry C.E., Tekaia F., Badcock K., Basham D., Brown D., Chillingworth T., Connor R., Davies R., Devlin K., Feltwell T., Gentles S., Hamlin N., Holroyd S., Hornsby T., Jagels K., Krogh A., McLean J., Moule S., Murphy L., Oliver K., Osborne J., Quail M.A., Rajandream M.A., Rogers J., Rutter S., Seeger K., Skelton J., Squares S., Squares R., Sulston J.E., Taylor K., Whitehead S., Barrel l B.G. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature vol. 393, p. 537-544, 1998.
39. Meissner G., Schroder, K.H. Amadio G.E., Anz W., Chaparas S., Engel H.W.B., Jenkins P.A., Kappler W., Kleeberg H.H., Kubala E., Kubin M., Lauterbach D., Lind A., Magnusson M., Mikova Z., Pattyn S.R., Schaefer W.B., Stanford J.L., Tsukamura M., Wayne L.G., Willers I., Wolinsky E.. A co-operative numerical analysis of nonscoto- and nonphotochromagenic slowly growing mycobacteria. Journal of General Microbiology. vol. 83, p. 207-235, 1974.

40. Wayne L.G., Good R.C., Krichevsky M.I., Beam R.E., Blacklock Z., Chaparas S.D., Dawson D., Froman S., Gross W., Hawkins J., Jenkins P.A., Juhlin I., Kappler W., Kleeberg H.H., Krasnow I., Lefford M.J., Mankiewicz E., McDurmont C., Meissner G., Morgan P., Nel E.E., Pattyn S.R., Portaels F., Richards P.A., Rusch S., Schroder K.H., Silcox V.A., Szabo I., Tsukamura M., Vergmann B. First report of the cooperative, openended study of slowly growing mycobacteria by the InternationalWorking Group on Mycobacterial Taxonomy. International Journal of Systematic Bacteriology. vol. 31, p. 1-20, 1981.
41. Grange J.M., Yates M.D., Boughton E.. The avian tubercle bacillus and its relatives. Journal of Applied Bacteriology. vol. 68, p. 411-431, 1990.
42. Thoresen O.F., Saxegaard F. Comparative use of DNA probes for Mycobacterium avium and Mycobacterium intracellulare and serotyping for identification and characterization of animal isolates of the M avium complex. Veterinary Microbiology. vol. 34, p. 83-88, 1993.
43. Ramasoota P., Chansiripornchai N., Kallenius G., Hoffner S.E., Svenson S.B. Comparison of Mycobacterium avium complex (MAC) strains from pigs and humans in Sweden by random amplified polymorphic DNA (RAPD) using standardized reagents. Veterinary Microbiology. vol. 78, p. 251-259, 2001.
44. Bezdicek P., Chrystal R.D. In: Chrystal R.D., West J.B., Weiberland E.R., Bames P.J. (Eds.), The Lung: Scientific Foundations, Lippincott–Rowan, Philadelphia, vol. 1, p. 577–588, 1997.
45. Crapo J.D. New concepts in pulmonary edema. Am. Rev. Resp. Dis. vol. 147, p. 790–792, 1993.
46. Crapo J.D., Bary B.E., Gehr P., Bachofen M., Weibel E.R. Cell numbers and characteristics of the human lung. Am. Rev. Res. Dis. vol. 125, p. 332–337, 1982.
47. Patton J.S. Mechanisms of macromolecule absorption by the lungs. Adv. Drug Deliv. Rev. vol. 19, p. 3–36, 1996.
48. Bezdicek P., Chrystal R.D. In: Chrystal R.D., West J.B., Weiberland E.R., Bames P.J. (Eds.), The Lung: Scientific Foundations, Lippincott–Rowan, Philadelphia. vol. 1, p. 577–588, 1997.
49. Brain JD. Mechanisms, measurement, and significance of lung macrophage function. Environ Health Perspect. Vol. 97 p. 5–10, 1992
50. Gehr P, Geiser M. Im Hof V, Schurch S, Waber U, Baumann M. Surfactant and inhaled particles in the conducting airways: structural, stereological, and biophysical aspects. Microsc Res Technol. vol. 26(6), p. 423–436, 1993.
51. Beck BD, Brain JD, Bohannon DE. An in vivo hamster bioassay to assess the toxicity of particulates for the lungs. Toxicol Appl Pharmacol. vol. 66(1), p. 9–29, 1982;
52. Lalor C, Atkins K, Concessio N, Pettis R, Hickey A. Lung deposition and alveolar macrophage uptake of microparticles from suspension aerosols in guinea pigs. Society of Toxicology, 35th Annual Meeting, Anaheim, CA; 1996.
53. Oberdorster G, Ferin J, Gelein R, Soderholm SC, Finkelstein J. Role of the alveolar macrophage in lung injury: studies with ultrafine particles. Environ Health Perspect. Vol. 97, p. 193–199, 1992.
54. Snipes MB, Clem MF. Retention of microspheres in the rat lung after intratracheal instillation. Environ Res. Vol. 24(1), p. 33–41, 1981.
55. Oberdorster G, Cox C, Gelein R. Intratracheal instillation versus intratracheal inhalation of tracer particles for measuring lung clearance function. Exp Lung Res. Vol. 23(1). P. 17–34, 1997.
56. Ferin J. Pulmonary retention and clearance of particles. Toxicol Lett. Vol. 72(1–3), p. 121-125, 1994.
57. Green GM, Jakab GJ, Low RB, Davids GS. Defense mechanisms of the respiratory membrane. Am Rev Respir Dis, vol. 115(3), p. 479-514, 1977.
58. Shepherd VL. The role of the respiratory burst of phagocytes in host defense. Semin Respir Infect. Vol. 1(2), p. 99-106, 1986.
59. Sugar AM, Field KG. Characterization of murine bronchoalveolar macrophage respiratory burst: comparison of soluble and particulate stimuli. J Leuko Biol. Vol. 44(6), p. 500–507, 1988.
60. Patton J. Mechanisms of macromolecule absorption by the lungs. Adv Drug Del Rev. vol. 19, p. 3-361, 1996.
61. Mygind N. Structure and function of the respiratory tract. In: Moren F, Newhouse M, editors. Aerosols in medicine. New York: Elsevier; 1985.

62. Wiebel E. The lung: scientific foundations. New York: Raven Press; 1991.
63. Mercer R.R., Russell M.L., Roggli V.L., Crapo J.D. Cell number and distribution in human and rat airways. Am J Respir Cell Mol Biol. Vol. 10(6), p. 613-624, 1994.
64. Weibel E. Morphometrics of the lung. Handbook of physiology, Washington DC: American Physiological Society. Vol 1; p. 285, 1964.
65. Yu J., Chien Y.W. Pulmonary drug delivery: physiologic and mechanistic aspects. Crit Rev Ther Drug Carrier Syst. Vol. 14(4), p. 395-453, 1997.
66. Batenburg J.J. Surfactant phospholipids: synthesis and storage. Am J Physiol. Vol. 262(4 Pt 1)p. L367-L385, 1992.
67. Hawgood S. Pulmonary surfactant apoproteins: a review of protein and genomic structure. Am J Physiol. Vol. 257(2 Pt 1), p. L13–L22, 1989.
68. Evander E., Wollmer P., Jonson B., Lachmann B. Pulmonary clearance of inhaled 99mTc-DTPA: effects of surfactant depletion by lung lavage. J Appl Physiol. Vol. 62(4), p.1611-1614, 1987.
69. Wollmer P., Evander E., Jonson B. Pulmonary clearance of inhaled 99m Tc-DTPA: effect of surfactant depletion in rabbits. Clin Physiol. Vol. 6(1), p. 85-89, 1986.
70. Sanderson M.J., Sleigh M.A. Ciliary activity of cultured rabbit tracheal epithelium: beat pattern and metachrony. J Cell Sci. vol. 47, p. 331-347, 1981.
71. Lucas A., Douglas L. Principles underlying ciliary activity in the respiratory tract. Arch Otolaryngol. Vol. 34, p. 495, 1934.
72. Jeffrey P.K. The origins of secretions in the lower respiratory trac. Eur J Respir Dis Suppl. Vol. 153, p. 34-42, 1987.
73. Lippmann M., Schlesinger R.B. Interspecies comparisons of particle deposition and mucociliary clearance in tracheobronchial airways. J Toxicol Environ Health. Vol. 13(2–3), p. 441-469, 1984.
74. Gupta P., Hickey A. Contemporary approaches in aerosolized drug delivery to the lung. J Control Rel. vol. 17, p. 129-148, 1991.
75. Boe J., Dennis J.H., O'Driscoll B.R., Bauer T.T., Carone M., Dautzenberg B. European Respiratory Society guidelines on the use of nebulizers. Eur Respir J. vol. 18(1), p. 228-242, 2001.
76. Finlay W.H. The mechanics of inhaled pharmaceutical aerosols, an introduction. London: Academic Press. chapter 8, 2001.
77. Hickey A.J., Martonen T.B. Behavior of hygroscopic pharmaceutical aerosols and the influence of hydrophobic additives. Pharm Res. Vol. 10(1), p. 1-7, 1993.
78. Mercer T.T., Tillery M.I., and Chow H.Y. Operating characteristics of some compressed-air nebulizers, Am. Ind. Hyg. Assoc. J. vol. 29, p. 66-78 1968.
79. Smaldone G.C., Perry R.J., and Deutsch D.G. Characteristics of nebulizers used in the treatment of AIDS-related Pneumocystis carinii pneumonia, J. Aerosol Med. vol. 1, p. 113-l26, 1988.
80. Sterk P.J., Plomb A., Van de Vate J.F., and Quanjer P.H. Physical properties of aerosols produced by several jet- and ultrasonic nebulizers, Bull. Eur. Physiopathol. Respir. Vol. 20, p. 65-72, 1984.
81. Ferron G.A., Weber J., and Kerrebijn K.F. Properties of aerosols produced with three nebulizers, Am. Rev. Respir. Dis. vol. 114, p. 899-908, 1976.
82. Porstendörfer J., Gebhart J., and Röbig G. Effect of evaporation on the size distribution of nebulized aerosols, J. Aerosol Sci. vol. 8, p. 371-380, 1977.
83. Mercer T.T., Goddard R.E, and Flores R.L., Output characteristics of several commercial nebu~izers, Ann. Allergy. Vol. 23, p. 314-326, 1965.
84. Mercer T.T., Production of therapeutic aerosols. Principles and techniques, Chest. Vol. 80 (suppl.), p. 813-818, 1981.
85. Phipps P.R., and Gonda I., Droplets produced by medical nebulizers: some factors affecting their size and solute concentration, Chest. Vol. 97, p. 1327-1332, 1990.
86. Schlesinger R.B. Clearance from the respiratory tract. Fundam Appl Toxicol. Vol. 5(3), p. 435–450, 1985.
87. Anderson P.J., Wilson J.D., Hiller F.C. Respiratory tract deposition of ultrafine particles in subjects with obstructive or restrictive lung disease. Chest. Vol. 97(5), p. 1115-1120, 1990.

88. Carpenter R.L. Aerosol deposition modeling using ACSL. Drug Chem Tox. Vol. 22(1), p. 73–90, 1999.
89. O'Doherty M.J., Miller R.F. Aerosols for therapy and diagnosis. Eur J Nucl Med. vol. 20(12), p. 1201-1213, 1993.
90. Yu J., Chien Y.W. Pulmonary drug delivery: physiologic and mechanistic aspects. Crit Rev Ther Drug Carrier Syst. Vol. 14(4), p. 395-453, 1997.
91. Schlesinger R.B. Comparative deposition of inhaled aerosols in experimental animals and humans: a review. J Toxicol Environ Health, vol. 15(2), p. 197-214, 1985.
92. Brain J.D., Valberg P.A. Deposition of aerosols in the respiratory tract (review). Am Rev Respir Dis. vol. 120(6), p. 1325-1373, 1979.
93. Lippmann M. Regional deposition of particles in the human respiratory tract handbook of physiology. American Physioligical Society; 1977.
94. Zainudin B.M. Therapeutic aerosol: principles and practices. Med J Malaysia. Vol. 48(3), p. 259-268, 1993.
95. Byron P.R., Patton J.S. Drug delivery via the respiratory tract. J Aerosol Med. vol. 7(1), p. 49-75, 1994.
96. Lippmann M., Schlesinger R.B. Interspecies comparisons of particle deposition and mucociliary clearance in tracheobronchial airways. J Toxicol Environ Health. Vol. 13(2–3), p. 441-469, 1984.
97. Byron P.R. Prediction of drug residence times in regions of the human respiratory tract following aerosol inhalation. J Pharm Sci. vol. 75(5), p. 433-438, 1986.
98. Hakkinen A.M., Uusi-Heikkila H., Jarvinen M., Saali K., Karhumaki L. The effect of breathing frequency on deposition of drug aerosol using an inhalation-synchronized dosimeter in healthy adults. Clin Physiol. Vol. 19(3), p. 269-274, 1999.
99. Gonda I. Aerosols for delivery of therapeutic and diagnostic agents to the respiratory tract. Crit Rev Ther Drug Carrier Syst. Vol. 6(4), p. 273-313, 1990.
100. Valberg P.A., Brain J.D., Sneddon S.L., Le Mott S.R. Breathing patterns influence aerosol deposition sites in excised dog lungs. J Appl Physiol. Vol. 53(4), p. 824-837, 1982.
101. Svartengren K., Philipson K., Svartengren M., Anderson M., Camner P. Tracheobronchial deposition and clearance in small airways in asthmatic subjects. Eur Respir J. vol. 9(6), p. 1123-1129, 1996.
102. Svartengren K., Ericsson C.H., Svartengren M., Mossberg B., Philipson K., Camner P. Deposition and clearance in large and small airways in chronic bronchitis. Exp Lung Res. Vol. 22(5), p. 555-576, 1996.
103. Oberdorster G. Lung clearance of inhaled insoluble and soluble particles. J Aerosol Med. vol. 1, p. 289-330, 1988.
104. Foster W.M., Langenback E.G., Bergofsky E.H.. Disassociation in the mucociliary function of central an peripheral airways of asymptomatic smokers. Am Rev Respir Dis. vol. 132(3), p. 633-639, 1985.
105. Messina M.S., O'Riordan T.G., Smaldone G.C.. Changes in mucociliary clearance during acute exacerbations of asthma. Am Rev Respir Dis. vol. 143(5 Pt 1), p. 993-997, 1991.
106. Svartengren M., Ericsson C.H., Philpson K., Mossberg B., Camner P. Tracheobronchial clearance in asthma-discordant monozygotic twins. Respiration. Vol. 56(1–2), p. 70-79, 1989.
107. Raviglione M.C., Snider D.E., and Kochi A. Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic. JAMA. Vol. 273, p. 220-226, 1995.
108. Shinnick T. Current Topics in Microbiology and Immunology (New York: Springer-Verlag) 1996.
109. Maggi N., Pasqualucci C. R., Ballotta R., Sensi P. Chernotherapia. Vol. 11, p. 285-292, 1966.
110. Sensi P. History of the development of rifampin. Rev. Infect. Dis. vol. 3, p. 402-40, 1983.
111. Hartmann G., Honikel K.O., Knusel F., Nuesch J. The specific inhibition of the DNA-directed RNA synthesis by rifamycin Biochim. Biophys. Acta. Vol. 145, p. 843-844, 1967.
112. Archambault J., Friesen J.D. Genetics of RNA polymerases I, II, and III. Microbiol. Rev. vol. 57, p. 703-724, 1993.

113. Ezekiel D.H., Hutchins J.E. Mutations affecting RNAP associated with rifampicin resistance in Escherichia coli. Nature London. Vol. 220, p. 276-277, 1968.
114. Wehrli W., Neusch J., Knusel F., Staehelin M. Action of rifamycin on RNA olymerase from sensitive and resistant bacteria. Biochem. Biophys. Res. Commun. Vol. 32, p. 284-288, 1968b.
115. Heil A., and Zillig W. Reconstitution of bacterial DNA-dependent RNA polymerase from isolated subunits as a tool for the elucidation of the role of the subunits in transcription. FEBS Lett. Vol. 11, p. 165-168, 1970.
116. Ramaswamy S., and Musser J.M. Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis: 1998 update. Tuber. Lung Dis. vol. 79, p. 3-29, 1998.
117. Heep M., Rieger U., Beck D., and Lehn N. Mutations in the beginning of the rpoB gene can induce resistance to rifamycins in both Helicobacter pylori and Mycobacterium tuberculosis. Antimicrob. Agents and Chemotherapeutics. Vol. 44, p. 1075-1077, 2000.
118. Ovchinnikov Y.A., Monastyrskaya G.S., Guriev S.O., Kalinina N.F., Sverdlov E.D. Gragerov, A.I. Bass, I.A. Kiver, I.F. Moiseyeva, E.P. Igumnov, V.N. et al. RNA polymerase rifampicin resistance mutations in Escherichia coli: sequence proved changes and dominance. Mol. Gen. Genet. Vol. 190, p. 344-348, 1983.
119. Lisitsyn N.A., Gur'ev S.O., Sverdlov E.D., Moiseeva E.P., and Nikiforov V.G. Nucleotide substitutions in the rpoB gene leading to rifampicin resistance of E. coli RNA polymerase. Bioorg Khim. Vol. 10, p. 127-128, 1984a.
120. Lisitsyn N.A., Sverdlov E.D., Moiseyeva E.P., Danilevskaya O.N., and Nikiforov V. Mutation to rifampicin resistance at the beginning of the RNA polymerase beta subunit gene in Escherichia coli. Mol. Gen. Genet. Vol. 196, p. 173-174, 1984b.
121. Jin D.J., and Gross C.A. Mapping and sequencing of mutations in the Escherichia coli rpoB gene that lead to rifampicin resistance. J. Mol. Biol. Vol. 202, p. 45-58, 1988.
122. Severinov K. Soushko, M. Goldfarb, A. and Nikiforov A. Rifampicin region revisited. New rifampicin-resistant and streptoly-digin-resistant mutants in the beta subunit of Escherichia coli RNA polymerase. J. Biol. Chem. Vol. 268, p. 14820-14825, 1993.
123. Severinov K., Soushko M., Goldfarb A., and Nikiforov V. RifR mutations in the beginning of the Escherichia coli rpoB gene. Mol. Gen. Genet. Vol. 244, p. 120-126, 1994.
124. Blomberg B., Evans, P. Phanouvong S., Nunn P. Informal consultation on 4-drug fixed dose combinations (4FDCs) compliant with the WHO model list of essential drugs. World Health Organization, Geneva, WHO/CDS/TB/2002. vol. 299, p. 15-17, 2002.
125. Grant D.J.W. Theory and origin of polymorphism. In: Brittain, H.G. (Ed.), Polymorphism in Pharmaceutical Solids. Marcel Dekker, New York. p. 1-34, 1999.
126. Pelizza G., Nebuloni M., Ferrari P., Gallo G.G. Polymorphism of rifampicin. Il Farmaco. Vol. 32, p. 471-481, 1977.
127. Henwood S.Q., Liebenberg W., Tiedt L.R., Lotter A.P., Villers M.M. Characterization of the solubility and dissolution properties of several new rifampicin polymorphs, solvates and hydrates. Drug Dev. Ind. Pharm. Vol. 27, p. 1017-1030, 2001.
128. Mathiowitz E., Langer R. Polyanhydride microspheres as drug carriers I. Hot melt microencapsulation. J. Control. Rel. vol. 5, p. 13-22, 1987.
129. Folkman J., Long D.M. The use of silicone rubber as a carrier for prolonged drug therapy. J. Surg. Res. Vol. 4, p. 139-142, 1964.
130. Desai S.J., Siminelli A.P., Higuchi W.I. Investigation of factors influencing release of solid drug dispersed in inert matrixes. J. Pharm. Sci. vol. 54, p. 1459-1464, 1965.
131. Jalil R., Nixon J.R. Biodegradable poly(lactic acid) and poly(lactide-co-glycocide) microcapsules: problems associated with preparative techniques and release properties. J. Microencapsul. Vol. 7, p. 297-325, 1990b.
132. Chang T.M.S. Semipermeable microcapsules. Science. Vol. 146, p. 524-525, 1964.
133. Mason N., Thies C., Cicero T.J. In-vivo and in vitro evaluation of a microencapsulated narcotic anagonist. J. Pharm. Sci. vol. 65, p. 847-850, 1976.
134. Jain R.A. The manufacturing techniques of various drug loaded biodegradable poly(lactide-coglycolide) (PLGA) devices. Biomaterials.vol. 21, p. 2475-2490, 2000.

135. Fukushima S.; Kishimoto S.; Takeuchi Y.; Fukushima M. Preparation and evaluation of o/w type emulsions containing antitumor prostaglandin. Adv. Drug. Deliv. Rev. vol. 45, p. 65-75, 2000.

136. Okada H.; Toguchi H. Biodegradable microspheres in drug delivery. Crit. Rev. Ther. Drug Carrier Syst. Vol.12, p. 1-99, 1995.

137. Yang Y., Chung T.S., Bai X.L., Chan W.K. Effect of preparation conditions on morphology and release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion method. Chem. Eng. Sci. vol. 55, p. 2223–2236, 2000.

138. Ghaderi R., Struesson C., Carlfors J. Effect of preparative parameters on the characteristics of poly(d,l-lactideco-glocolide) microspheres made by the double emulsion method. Int. J. Pharm. Vol. 141, p. 205–216, 1996.

139. Yan C., Resau J.H., Hewetson J.,West M., Rill W.L., Kende M. Characterization and morphological analysis of protein loaded poly(lactide-co-glycolide) microparticles prepared by water-in-oil–in-water emulsion technique. J. Control Release. Vol 32, p. 231-241, 1994.

140. McGee J.P., Singh M., Li X.M., Qui H., O'Hagen D.T. The encapsulation of a model protein in poly(d,l-lactideco-glycocide) microparticles of various sizes: an evaluation of process reproduciblity. J. Microencapsul. Vol. 14, p. 197-210, 1997.

141. Schlicher E.J.A.M., Postma N.S., Zuidema J., Talsma H., Hennink W.E. Preparation and characterisation of poly(d,l-lactic-co-glycolic acid) microspheres containing desferrioxamine. Int. J. Pharm. Vol. 153, p. 235-245, 1997.

142. Lin Y.H.E., Vasavada R.C. Studies on microencapsulation of 5-fluorouracil with poly(ortho ester) polymers. J. Microencapsul. Vol. 17, p. 1–11, 2000.

143. Jeyanthi R., Thanoo B.C., Metha R.C., DeLuca P.P. Effect of solvent removal technique on the matrix characteristics of polylactide/glcolide microspheres for peptide delivery. J. Control Release. Vol. 38, p. 235-244, 1996.

144. Jalil R., Nixon J.R. Microencapsulation using poly(l-lactic acid) II: preparative variables affecting microcapsule properties. J. Microencapsul. Vol. 7, p. 25-39, 1990a.

145. LeCorre, P. LeGuevello, P. Gajan, V. Chevanne, F. LeVerge, R. Preparation and characterization of bupivacaine-loaded polylactide and polylactide-co-glycolide microspheres. Int. J. Pharm. Vol. 107, p. 41-49, 1994.

146. Ghaderi R., Struesson C., Carlfors J. Effect of preparative parameters on the characteristics of poly(d,l-lactideco-glocolide) microspheres made by the double emulsion method. Int. J. Pharm. Vol. 141, p. 205-216, 1996.

147. Mogi T., Ohtake N., Yoshida M., Chimura R., Kamaga Y., Ando S., Tsukamoto T., Nakajima T., Uenodan H., Otsuka M., Matsuda Y., Ohshima K., Makino K. Sustained release of 17 β-estradiol from poly(lactide-co-glycocide) microspheres in vitro and in vivo. Colloid Surface B. vol. 17, p. 153-165, 2000.

148. Narayani R., Rao K.P. Controlled release of anticancer drug methotrexate from biodegradable gelatin microspheres. J. Microencapsul. Vol. 11, p. 69-77, 1994.

149. Narayani R., Rao K.P. pH responsive gelatin microspheres for the oral delivery of anticancer drug methotrexate. J. Appl. Polym. Sci. vol. 58, p. 1761-1769, 1995.

150. Akhtar S., Lewis K. Antisense oligonucleotide delivery to cultured macrophages is improved by incorporation into sustained-release biodegradable polymer microspheres. Int. J. Pharm. Vol. 151, p. 57–67, 1997.

151. Sansdrap P., Moes A.J. In vitro evaluation of the hydrolytic degradation of dispersed and aggregated poly(d,l-lactide-co-glycolide) microspheres. J. Control Release. Vol. 43, p. 47-58, 1997.

152. Bezemer J.M., Radersma R., Grijpma D.W., Dijkstra P.J., Blitterswijk C.A.V., Feijen J. Microspheres for protein delivery prepared from amphiphilic multiblock copolymers 2. Modulation of release rate. J. Control Release. Vol. 67, p. 249-260, 2000.

153. Ermis D., Yuksel A. Preparation of spray-dried microspheres of indomethacin and examination of the effects of coating on dissolution rates. J. Microencapsul. Vol. 16, p. 315-324, 1999.

154. Huang Y.Y., Chung T.W., Tzeng T.W. A method using biodegradable polylactides/polyethylene glycol for drug release with reduced initial burst. Int. J. Pharm. Vol. 182, p. 93-100, 1999.
155. Jones C.D., Lyon L.A. Synthesis and characterization of multiresponsive core-shell microgels. Macromolecules. Vol. 33, p. 8301-8306, 2000.
156. Lee T.H., Wang J., Wang C.H. Double-walled microspheres for the sustained release of a highly water soluble drug: characterization and irradiation studies. J. Control Release. Vol. 83, p. 437-452, 2002.
157. Sparnacci K., Laus M., Tondelli L., Magnani L., Bernardi C. Core-shell microspheres by dispersion polymerization as drug delivery systems. Macromol. Chem. Phys. Vol. 203, p. 1364-1369, 2002.
158. Zhou S., Deng, X. Yuan, M. Li X. Investigation on preparation and protein release of biodegradable polymer microspheres as drug-delivery system. J. Appl. Polym. Sci. vol. 84, p. 778-784, 2002.
159. Mathiowitz E., Chickering D.E., Jacob J.S. US Pat. No. 6,197,346, 2001.
160. Sakagami M., Kinoshita W., Sakon K., Sato J., Makino Y. Mucoadhesive beclomethasone microspheres for powder inhalation: their pharmacokinetics and pharmacodynamics evaluation, J. Control. Release. Vol. 80 p. 207-218, 2002.
161. Sakagami M., Sakon K., Kinoshita W., Makino Y. Enhanced pulmonary absorption following aerosol administration of mucoadhesive powder microspheres, J. Control. Release. Vol. 77, 117-129, 2001.
162. Borchard G., Luegen H.L., de Boer A.G., Verhoef J.C., Lehr C.M. Junginger H.E. The potential of mucoadhesive polymers in enhancing intestinal peptide drug absorption: III. Effects of chitosan-glutamate and carbomer on epithelial tight junctions in vitro, J. Control. Release 39 (1996) 131– 138.
163. Takeuchi H., Matsui Y., Yamamoto H., Kawashima Y., Mucoadhesive properties of carbopol or chitosan-coated liposomes and their effectiveness in the oral administration of calcitonin to rats, J. Control. Release. Vol. 86, p. 235-242, 2003.
164. Herrero-Vanrell R., Fernandez-Carballido A., Frutos G., Cadorniga R. Enhancement of the mydriatic response to tropicamide by bioadhesive polymers, J. Ocular Pharmacol. Ther. Vol.16, p. 419-428, 2000.
165. Lim S.T., Forbes B., Martin G.P., Brown M.B. In vivo and in vitro characterization of novel microparticulates based on hyaluronan and chitosan hydroglutamate, AAPS PharmSciTech. Vol. 2, p. 20, 2001.
166. Thompson D.C. Pharmacology of therapeutic aerosols. In Hickey, A.J. (Ed.), Pharmaceutical Inhalation Aerosol Technology, Dekker, New York, p. 29-59, 1992.
167. Byron P.R., Prediction of drug residence time, in region of the human respiratory tract following aerosol inhalation. J. Pharm. Sci. vol. 75 p. 433-438, 1986.
168. Malo J.L., Cartier A., Merland N., Ghezzo It., Burek A., Morris J. and Jennings B.H. Four-times-a-day dosing frequency is better than a twice-a-day regimen in subjects requiring a high-dose inhaled steroid, budesonides to control moderate to severe asthma. Am. Rec. Respir. Dis. vol. 140, p. 624-628, 1989.
169. Patton J.S. and Platz R.M. Pulmonary delivery of peptides and proteins for systemic action. Adv. Drug Del. ReL. vol. 8 p. 179-196, 1992.
170. Edwards D.A., Abdelaziz B.J., Langer R. Recent advances in pulmonary drug delivery using large, porous inhaled particles. J Appl Phys. Vol. 85, p. 379-85, 1998.
171. Gupta P.K. and Hung C.T., Albumin microspheres: I. Physico-chemical characteristics. J. Microencapsul. vol. 4, p. 427-462, 1989.
172. Zeng X.M., Martin G.P. and Marriott C., Tetrandrine delivery to the lung: the optimisation of the preparation of albumin microspheres by central composite design. Int. J. Pharm. Vol. 109, p. 135-145, 1994.

173. Zeng X.M., Martin G.P. and Marriott C. The preparation and in vitro evaluation of tetrandrine-entrapped albumin microspheres as an inhalation drug delivery system. Eur. J. Pharm. Sci. vol. 3 p. 87-93, 1995.
174. Tice T.R., Mason D.W. and Gilley R.M. Clinical use and future of parenteral microsphere delivery systems. In Prescott, L.F. and Nimmo, W.S. (Eds), Novel Drug Delivery and its Therapeutic Application, Wiley, New York. p. 223-235, 1989.
175. Masinde L. and Hickey A.J. Nebulization of poly(lactic acid) microspheres from aqueous suspension. Pharm. Res. Vol. 8 p. 120, 1991.
176. Lai Y.L., Mehta R.C., Thacker A.A., Yoo S.-D., McNamara P.J. and Deluca P.P. Sustained bronchodilation with isoproterenol poly(glycolide-co-lactide) microspheres. Pharm. Res. Vol. 10, p. 119-125, 1993.
177. Edwards D.A., Hanes J., Caponetti G., Hrkach J., Ben Jebria A., Eskew M.L., Mintzes J., Deaver D., Lotan N., Langer R. Large porous particles for pulmonary drug delivery. Science. Vol. 276, p. 1868-1871, 1997.
178. O'Hara P., Hickey A.J. Respirable PLGA microspheres containing rifampicin for the treatment of tuberculosis: Manufacture and characterization. Pharm Res. Vol. 17, p. 955-961, 2000.
179. O'Hara P. and Hickey A.J. Respirable PLGA Microspheres Containing Rifampicin for the Treatment of Tuberculosis: Manufacture and Characterization Pharmaceutical Research, Vol. 17/8, 2000.
180. Pandey R., Khuller G.K. Chemotherapeutic potential of alginate–chitosan microspheres as antitubercular drug carriers. J Antimicrob Chemother. vol. 53, p. 635-40, 2004.
181. Tonnesen H.H., Karlsen J. Alginate in drug delivery systems. Drug Dev Ind Pharm. Vol. 28, p. 621-30, 2002.
182. Mukherjee J.S., Rich M.L., Socci A.R. et al.. Programmes and principles in treatment of multidrug-resistant tuberculosis. Lancet. Vol. 363, p. 474-81, 2004.
183. Muzzarelli R.A.A. Chitin. Pergamon Press, New York. p. 1-37, 1977.
184. Roberts G.A.F. Chitin Chemistry. Mac Millian Press, Houndmills. p. 1-50, 1992.
185. Kas H.S. Chitosan: properties, preparation and application to microparticulate systems. J. Microencapsul. Vol. 14, p. 689-711, 1997.
186. Singla A.K., Sharma M.L., Dhawan S. Nifedipine loaded chitosan microspheres: characterization of internal structure. Biotech. Histochem. Vol. 76, p. 165-171, 2001.
187. Kato Y., Onishi H., Machida Y. Application of chitin and chitosan derivatives in the pharmaceutical field. Curr. Pharm. Biotechnol. Vol. 4, p. 303-309, 2003.
188. Fukuda H. Polyelectrolyte complexes of chitosan carboxymethyl cellulose. Bull. Chem. Soc. Jpn. Vol. 53, p. 837-840, 1980.
189. Chandy T., Sharma C.P. Chitosan as a biomaterial biomater. Artif. Cells Artif. Organs. Vol. 18, p. 1-24, 1990.
190. Illum L., Jabbal-Gill I., Hinchcliffe M., Fisher A.N., Davis S.S. Chitosan as a novel nasal delivery system for vaccines. Adv. Drug Deliv. Rev. vol. 51, p. 81-96, 2001.
191. Furda I. Nonabsorbable lipid binder. US Patent 4,223,023, 1980.
192. Nagyuvary J.J. Treating hyperbilirubinemia. US Patent 4,363,801, 1982.
193. Tachihara K., Onishi H., Machida Y. Preparation of silver sulfadiazine-containing spongy membranes of chitosan and chitin–chitosan mixture and their evaluation as burn wound dressings. Arch. Practical Pharm. Vol. 57, p. 159-167, 1997.
194. Felt O., Furrer P., Mayer J.M., Plazonnet B., Buri P., Gurny R. Topical use of chitosan in ophthalmology: tolerance assessment and evaluation of precorneal retention. Int. J. Pharm. Vol. 180, p. 185-193, 1999.
195. Kristmundsdottir T., Ingvarsdottir K., Saemundsdottir G. Chitosan matrix tablets: the influence of excipients on drug release. Drug Dev. Ind. Pharm. Vol. 21, p. 1591-1598, 1995.
196. Sabnis S., Rege P., Block L.H. Use of chitosan in compressed tablets of diclofenac sodium: inhibition of drug release in an acidic environment. Pharm. Dev. Technol. vol. 2, p. 243-255, 1997.

197. Illum L. Chitosan and its use as a pharmaceutical excipient. Pharm. Res. Vol. 15, p. 1326-1331, 1998.
198. Upadrashta S.M., Katikaneni P.R., Nuessle N.O. Chitosan as a tablet binder. Drug Dev. Ind. Pharm. Vol. 18, p. 1701-1708, 1992.
199. Ilango R., Kavimani S., Premila J., Nair D.K., Jayakar B. Studies on tablets of sulphamethoxazole using chitosan. Ind. J. Pharm. Sci. vol. 59, p. 220-224, 1997.
200. Hou W.M., Miyazaki S., Takada M., Komai T. Sustained release of indomethacin from chitosan granules. Chem. Pharm. Bull. Vol. 33, p. 3986–3992, 1985.
201. Kawashima Y., Handa T., Kasai A., Takenaka H., Lin S.Y. The effects of thickness and hardness of the coating film on the drug release rate of theophylline granules coated with chitosan–sodium tripolyphosphate complex. Chem. Pharm. Bull. Vol. 33, p. 2469-2474, 1985a.
202. Miyazaki T., Komuro T., Yomota C., Okada S. Use of chitosan as a pharmaceutical material effectiveness as an additional additive of sodium alginate. Eisei Shikenjo Hokoku. Vol. 108, 95-97, 1990.
203. Akbuga J. Effect of the physicochemical properties of a drug on its release from chitosan malate matrix tablets. Int. J. Pharm. Vol. 100, p. 257-261, 1993.
204. Kristl J., Smidx-Korbar J., Struc E., Schara M., Rupprecht H. Hydrocolloids and gels of chitosan as drug carriers. Int. J. Pharm. Vol. 99, p. 13-19, 1993.
205. Miyazaki S., Ishii K., Nadai T. Use of chitin and chitosan as drug carriers. Chem. Pharm. Bull. Vol. 29, p. 3067-3069, 1981.
206. Sawayanagi Y., Nambu N., Nagai T. Enhancement of dissolution properties of griseofulvin from ground mixtures with chitin and chitosan. Chem. Pharm. Bull. Vol. 30, p. 4464-4467, 1982a.
207. Shiraishi S., Arahira M., Imai T., Otagiri M. Enhancement of dissolution rates of several drugs by low-molecular weight chitosan and alginate. Chem. Pharm. Bull. Vol.38, p. 185-187, 1990.
208. Lehr C.M., Bouwstra J.A., Schacht E.H., Junginger H.E. In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. Int. J. Pharm. Vol. 78, p. 43-48, 1992.
209. He P., Davis S.S., Illum L. In vitro evaluation of the mucoadhesive properties of chitosan microspheres. Int. J. Pharm. Vol 166, p. 75-88, 1998.
210. Kockisch S., Rees G.D., Young S.A., Tsibouklis J., Smart J.D. Polymeric microspheres for drug delivery to the oral cavity: an in vitro evaluation of mucoadhesive potential. J. Pharm. Sci. vol. 92, p. 1614-1623, 2003.
211. Schipper N.G.M., Olsson S., Hoogstraate J.A., de Boer A.G., Varum K.M., Artursson P. Chitosans as absorption enhancers for poorly absorbable drugs. 2: Mechanism of absorption enhancement. Pharm. Res. Vol. 14, p. 923-929, 1997.
212. Dodane V., Khan M.A., Merwin J.R. Effect of chitosan on epithelial permeability and structure. Int. J. Pharm. Vol. 182, p. 21-32, 1999.
213. Schipper N.G.M., Varum K.M., Artursson P. Chitosan as absorption enhancers for poorly absorbable drugs. 1: Influence of molecular weight and degree of acetylation on drug transport across human epithelial (Caco-2) cells. Pharm. Res. Vol. 13, p. 1686-1692, 1996.
214. Lueen H.L., Lehr C.M., Rentel C.O., Noach A.B.J., de Boer A.G., Verhoef J.C., Junginger H.E. Bioadhesive polymers for the peroral delivery of peptide drugs. J. Control Rel. vol. 29, p. 329-338, 1994.
215. Soane R.J., Frier M., Perkins A.C., Jones N.S., Davis S.S., Illum L. Evaluation of the clearance characteristics of bioadhesive systems in humans. Int. J. Pharm. Vol. 178, p. 55-65, 1999.
216. Gallo J.M., Hassan E.E. Receptor-mediated magnetic carriers: basis for targeting. Pharm. Res. Vol. 5, p. 300-304, 1988.
217. Orienti I., Aiedeh K., Gianasi E., Bertasi V., Zecchi V. Indomethacin loaded chitosan microspheres. Correlation between the erosion process and release kinetics. J. Microencapsul. Vol. 13, p. 463-472, 1996.
218. Nishioka Y., Kyotani S., Okamura M., Miyazaki M., Okazaki K., Ohnishi S., Yamamoto Y., Ito K. Release characteristics of cisplatin chitosan microspheres and effect of containing chitin. Chem. Pharm. Bull. Vol. 38, p. 2871-2873, 1990.

219. Berthold A., Cremer K., Kreuter J. Preparation and characterization of chitosan microspheres as drug carrier for prednisolone sodium phosphate as model for anti-inflammatory drugs. J. Control Rel. vol. 39, p. 17-25, 1996a.
220. Berthold A., Cremer K., Kreuter J. Influence of crosslinking on the acid stability and physicochemical properties of chitosan microspheres. STP Pharm. Sci. vol. 6, p. 358-364, 1996b.
221. Hejazi R., Amiji M. Stomach-specific anti-H. pylori therapy. I: preparation and characterization of tetracylineloaded chitosan microspheres. Int. J. Pharm. Vol. 235, p. 87-94, 2002.
222. Jameela S.R., Kumary T.V., Lal A.V., Jayakrishnan A. Progesterone-loaded chitosan microspheres: a long acting biodegradable controlled delivery system. J. Control Rel. vol. 52, p. 17-24, 1998.
223. Kumar V., Lewis S.A., Mutalik S., Shenoy D.B., Udupa N. Biodegradable microspheres of curcumin for treatment of inflammation. Indian J. Physiol. Pharmacol. vol. 46, p. 209-217, 2002.
224. Dini E., Alexandridou S., Kiparissides C. Synthesis and characterization of cross-linked chitosan microspheres for drug delivery applications. J. Microencapsul. Vol. 20, p. 375-385, 2003.
225. Tsapis N., Bennett D., O'Driscoll K. et al. Direct lung delivery of para-aminosalicylic acid by aerosol particles. Tuberculosis (Edinburgh, Scotland). Vol. 83, p. 379-85, 2003.
226. Athanasiou K.A., Niederauer G.G., Agrawal C.M. Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid/polyglycolic acid copolymers. Biomaterials. Vol. 17, p. 93-102, 1996.
227. Göpferich A. Mechanisms of polymer degradation and erosion. Biomaterials. Vol. 17, p. 103-114, 1996a.
228. Göpferich A. Polymer degradation and erosion: mechanisms and application. Eur. J. Pharm. Biopharm. Vol. 42, p. 1-11, 1996b.
229. Husmann M., Schenderlein S., Lück M., Lindner H., Kleinebudde P. Polymer erosion in PLGA microparticles produced by phase separation method. Int. J. Pharm. Vol. 242, p. 277-280, 2002.
230. Okada H. One- and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate. Adv. Drug. Del. Rev. vol. 28, p. 43-70, 1997.
231. Hausberger A.G., DeLuca P.P. Characterization of biodegradable poly(D,L-lactide-coglycolide) polymers and microparticles. J. Pharm. Biomed. Anal. Vol. 13, p. 747-760, 1995.
232. Jain R.A. The manufacturing techniques of various drug loaded biodegradable poly(lactide-coglycolide) (PLGA) devices. Biomaterials. Vol. 21, p. 2475-2490, 2000d.
233. Jamshidi K., Hyon S.H., Ikada Y. Thermal characterization of polylactides. Polymer. vol. 29, p. 2229-2234, 1988.
234. Park T.G. Degradation of poly(D,L-lactic acid) microspheres: effect of molecular weight. J. Control. Release. Vol. 30, p. 161-173, 1994.
235. Li S. Hydrolytic degradation characteristics of aliphatic polyesters derived from lactic and glycolic acids. J. Biomed. Mater. Res. Vol. 48, p. 342-353, 1999.
236. Ogawa Y., Yamamoto M., Takada S., Okada H., Shimamoto T. Controlled-release of leuprolide acetate from polylactic acid or copoly(lactic / glycolic) acid microcapsules: influence of molecular weight and copolymer ratio of polymer. Chem. Pharm. Bull. Vol. 36, p. 1502-1507, 1988.
237. Omelczuk M.O., McGinity J.W. The influence of polymer glass transition temperature and molecular weight on drug release from tablets containing poly(DL-lactic acid). Pharm. Res. Vol. 9, p. 26-32, 1992.
238. Bodmeier R., Oh K.H., Chen H. The effect of the addition of low molecular weight poly(DLlactide) on drug release from biodegradable poly(DL-lactide) drug release systems. Int. J. Pharm. Vol. 51, p. 1-8, 1989.
239. Wada R., Tabata Y., Ikada Y. Preparation of poly(lactic acid) microspheres containing anti-cancer agents. Bull Inst Chem Res. Vol. 66, p. 241-250, 1988.
240. Okada H., Yamamoto M., Heya T., Inoue Y., Kamei S., Ogawa Y., Toguchi H. Drug delivery using biodegradable microspheres. J Controlled Release. Vol. 28, p. 121-129, 1994.

241. Csernus V.J., Szende B., Schally A.V. Release of peptides from sustained delivery systems (microcapsules and microparticles) in vivo. Int J Peptide Protein Res. Vol. 35, p. 557-565, 1990.
242. Wada R., Hyon S.H., Ikada Y. Lactic acid oligomer microspheres containing an anticancer agent for selective lymphatic delivery: I. In vitro studies. J Bioactive and Compatible Polymers. Vol. 3, p. 126-136, 1988.
243. Shenderova A., Burke T.G., Schwendeman S.P. Stabilization of 10- hydroxycamptothecin in poly(lactide-co-glycolide) microsphere delivery vehicles. Pharmaceut Res. Vol. 14, p. 1406-1414, 1997.
244. Wang Y.M., Sato H., Horkoshi I. In Vitro in vivo evaluation of taxol release from poly(lactic-co-glycolic acid) microspheres containing isopropyl myristate and degradation of the microspheres. J Controlled Release, vol. 49, p. 157-166, 1997.
245. Vook N., et al. Taxol microencapsulation into biodegradable uncapped poly(lactide-co-glycolide) copolymers. Int Symp Control Rel Bioact Mater. Vol. 24, p. 881-882, 1997.
246. Quenelle D.C., Winchester G.A., Staas J.K. Treatment of tuberculosis using a combination of sustainedrelease Rifampin-loaded microspheres and oral dosing with Isoniazid. Antimicrob. Agents Chemother. vol. 45, p. 1637-1644, 2001.
247. Rang H.P., Dale M.M., Rither J.M. Drugs used to treat tuberculosis. In: Pharmacology, fourth ed. Churchill Livingstone, London. p. 703-707, 1999.
248. Mandell G.L., Sande H.A. Antimicrobial agents. In: Gilman, G.A., Goodman, A. (Eds.), The Pharmacological Basis of Therapeutics, 7th ed. Macmillan, New York, p. 1202-1205, 1985.
249. Pandey, R., Zahoor, A., Sharma, S., Khuller, G.K. Nanoparticle encapsulated antitubercular drugs as a potential oral drug delivery system againstmurine tuberculosis. Tuberculosis.vol. 83, p. 373-378, 2003.
250. Jain C.P., Vyas S.P. Preparation and characterizationof niosomes containing rifampicin for lung targeting. J. Microencapsul. Vol. 12, p. 401–407, 1995.
251. Nakhare S., Vyas S.P. Multiple emulsion based systems for prolonged delivery of rifampicin: in vitro and in vivo characterization. Die Pharmazie. Vol. 52, p. 224-226, 1997.
252. Csaba N; González L; Sánchez A; Alonso M J. Design and characterisation of new nanoparticulate polymer blends for drug delivery J Biomat Scienc. Polymer Edition, vol. 15/9, p. 1137-1151, 2004
253. Bramwell V.W; Perrie Y. Particulate delivery systems for vaccines. Critical Reviews in Therapeutic Drug Carrier Systems. Vol. 22/2,p. 151-214, 2005
254. Sharma A., Pandey R., Sharma S., Khuller G.K. Chemotherapeutic efficacy of poly (dl-lactide-co-glycolide) nanoparticle encapsulated antitubercular drugs at sub-therapeutic dose against experimental tuberculosis. Int. J. of Antimicrobial Agents. Vol. 24, p. 599-604, 2004.
255. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye-binding. Anal Biochem. Vol. 72, p. 248-254, 1976.
256. Mosmann T.J. Rapid colorimetric assay for cellular growth and survival- Application to proliferation and cytotoxicity assays. Immunol. Methods. Vol. 65, p. 5, 1983.
257. Bridges P.A., Taylor K.M.G. An investigation of some of the factors influencing the jet nebulisation of liposomes. Int J Pharm. Vol. 204, p. 69-79, 2000.
258. Patel V.R., Amiji M.M. Preparation and characterization of freeze-dried chitosanpoly (ethylene oxide) hydrogels for site-specific antibiotic delivery in the stomach. Pharm. Res. Vol.13, p. 588-593, 1996.
259. Genta I., Costantini M., Asti A., Conti B., Montanari L. Influence of glutaraldehyde on drug release and mucoadhesive properties of chitosan microspheres. Carbohydrate Polymers. Vol. 36, p. 81-88, 1998.
260. Kawashim a Y., Yamamoto H., Takeuchi H., Hino T., Niwa T. Properties of a peptide containing dl-lactide/glycolide copolymer nanospheres prepared by novel emulsion solvent diffusion methods, Eur. J. Pharm. Biopharm. Vol. 45 p. 41-48, 1998.
261. Denis-Mize K.S., Dupuis M., MacKichan M.L., Singh M., Doe B., O'Hagan D., Ulmer J.B., Donnelly J.J., McDonald D.M., Ott G. Plasmid DNA adsorbed onto cationic microparticles

mediates target gene expression and antigen presentation by dendritic cells, Gene Ther. Vol. 7/24 p. 2105-2112, 2000.

262. Chiou S.H., Wu W.T., Huang Y.Y., Chung T.W. Effects of the characteristics of chitosan on controlling drug release of chitosan coated PLLA microspheres, J. Microencapsul. Vol. 18/5, p. 613-625, 2001.

263. Mandal B., Kempf M., Merkle H.P., Walter E. Immobilisation of GMCSF onto particulate vaccine carrier systems, Int. J. Pharm. Vol. 269/1, p. 259-265, 2004.

264. Chandy T., Das G.S., Rao G.H., 5-Fluorouracil-loaded chitosan coated polylactic acid microspheres as biodegradable drug carriers for cerebral tumours, J. Microencapsul. Vol. 17/5 p. 625-638, 2000.

265. Kumar M.N.V.R., Bakowsky U., Lehr C.M. Preparation and characterization of cationic PLGA nanospheres as DNA carriers, Biomaterials. Vol. 25/10, p. 1771-1777, 2004.

266. Oster C.G., Kim N., Grode L., Barbu-Tudoran L., Schaper A.K., Kaufmann S.H., Kissel T. Cationic microparticles consisting of poly(lactideco-glycolide) and polyethylenimine as carriers systems for parental DNA vaccination, J. Control. Release. Vol. 104/2 p. 359-377, 2005.

267. Walter E., Merkle H.P. Microparticle-mediated transfection of nonphagocytic cells in vitro, J. Drug Target. Vol. 10/1 p. 11-21, 2002.

268. O'Hagan D., Singh M., Ugozzoli M., Wild C., Barnett S., Chen M., Schaefer M., Doe B., Otten G.R., Ulmer J.B. Induction of potent immune responses by cationic microparticles with adsorbed human immunodeficiency virus DNA vaccines, J. Virol. Vol. 75/19 p. 9037-9043, 2001.

269. O'Hagan D., Singh M., Dong C., Ugozzoli M., Berger K., Glazer E., Selby M., Wininger M., Ng P., Crawford K., Paliard X., Coates S., Houghton M. Cationic microparticles are a potent delivery system for a HCV DNA vaccine, Vaccine. Vol. 23/5, p. 672-680, 2004.

270. Vila A., Sanchez A., Tobio M., Calvo P., Alonso M.J. Design of biodegradable particles for protein delivery, J. Control. Release. Vol. 78/1-3, p. 15-24, 2002.

271. Illum L., Farraj N.F., Davis S.S. Chitosan as a novel nasal delivery system for peptide drugs, Pharm. Res. Vol. 11/8 p. 1186-1189, 1994.

272. van der Lubben I.M., Verhoef J.C., Borchard G., Junginger H.E. Chitosan and its derivatives in mucosal drug and vaccine delivery, Eur. J. Pharm. Sci. vol. 14/3 p. 201-207, 2001.

273. Ravi Kumar M.N.V., Mohapatra S.S., Kong X., Jena P.K., Bakowsky U., and Lehr C.-M. Cationic Poly(lactide-co-glycolide) Nanoparticles as Efficient in vivo Gene Transfection Agents. Journal Of Nanoscience And Nanotechnology. Vol. 4/8, p. 1-5, 2004.

274. deBoer A.H., Molema G., Frijlink H.W., in: Molema G., Meijer D.K.F. (Eds.). Methods and Principles in Medical Chemistry. vol. 12, p. 53-87, 2001.

275. Takeuchi H., Shimotori Y., Yamamoto H., Hino T., Kawashima Y. Protective effects of chitosan and liposomal formulation for enzymatic degradation of insulin and their absorption promoting effects in intestinal mucous layer, Drug Deliv. Syst. Vol. 12, p. 347-352, 1997.

276. Yang T., Hussain A., Paulson J., Abbruscato T.J., Ahsan F. Cyclodextrins in nasal delivery of low-molecular-weight heparins: in vivo and in vitro studies. Pharm. Res. Vol. 21/7, p. 1127-1136, 2004.

277. Ohtake K., Maeno T., Ueda H., Natsume H., Morimoto Y. Poly-l-arginine predominantly increases the paracellular permeability of hydrophilic macromolecules across rabbit nasal epithelium in vitro, Pharm. Res. Vol. 20/2, 153-160, 2003.

278. Makino K., Nakajima T., Shikamura M., Ito F., Ando S., Kochi C., Inagawa H., Soma G.I., Terada H. Efficient intracellular delivery of rifampicin to alveolar macrophages using rifampicin-loaded PLGA microspheres: effects of molecular weight and composdition of PLGA on release of rifampicin. Coll and Surf B: Biointerfaces. vol 36, p. 35-42, 2004

279. Darwis Y., Kellaway,I.W. Nebulization of rehydrated freeze-dried beclomethasone dipropionate liposomes. Int. J. Pharm. Vol. 215, p. 113-121, 2001.