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Preparation and Optimization of Mannosylated Chitosan Microspheres by Modified Method for Pulmonary Delivery of Antitubercular Drugs

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Abstract: Objective: The aim of the present study is development and characterization of bioresponsive mannosylated chitosan microspheres (MCMs) for inhalational delivery of antitubercular drug.
Methods: Chitosan microsphere (CMs) prepared by modified ionic gelation method. We are here use ascorbic acid in place of acetic acid because L-ascorbic acid also have antioxidant activity. Then optimized the prepared carrier system for various formulation and process variables i.e. effect of chitosan and TPP concentration, pH of TPP solution and stirring time on particle size, entrapment efficiency and PDI to achieve desired size of pulmonary delivery of drug. Mannosylated chitosan microspheres prepared by incubation method and final carrier system characterized for drug delivery.
Results: The results of the particles size and surface characteristics showed the spherical shape and 1 - 5μ size of CMs and MCMs which can be suitable for pulmonary drug delivery. From in vitro drug release studies done using simulated lung fluids of RIF provided the advantage of controlled release characteristics deep inside the lung where tubercular bacilli reside and as suitable for pulmonary drug delivery it may help in improving treatment of tuberculosis through direct administration to site of action.

Keywords: Chdoi:10.1093/nar/17.17.6915itosan, Alveolar Macrophage Targeting, Antioxidant Property

I Introduction

Tuberculosis (TB) is caused by Mycobacterium tuberculosis (Mtb), an intracellular parasitic pathogen present in alveolar macrophages (AMs) that mainly affects the respiratory tract [1]. TB bacilli reside and proliferate within lung macrophages, the very cells that have evolved to engulf and destroy microorganisms that reach the surface of the lungs along with inhaled air [2]. Isoniazid (INH), Rifampicin (RIF), Pyrazinamide (PYZ), and Ethambutol (ETB) are the essential first line drugs used for the clinical disease management of TB. Treatment of TB remains a challenge for clinicians, because of the oral uptake of high systemic doses of single or combined antibiotics, which causes many side effects due to high systemic exposure. Thus, in spite of the availability of a wide variety of antibiotics that are active in-vitro, therapeutic failures are reported, mainly because of the inability of these drugs in exerting their potential activity against pathogens in an intracellular environment. Limitations exist in TB chemotherapy such as non-localised delivery of drugs, high dose and dose frequency as well as the adverse side effects that the therapy presents. To address these challenges various groups have reported the encapsulation of antituberculosis drugs where slow release, improved intracellular delivery and high drug loading parameters can be achieved by encapsulation in carrier system [3, 4].

Delivery of antiTB drugs directly to the primary infection site through pulmonary route may help in reduction of side effects as well as toxic effects and provide an advantage of dose reduction. Because of the increasing incidence of pulmonary diseases with high mortality and morbidity pulmonary drug delivery is emerging as a non-invasive and attractive approach for the treatment of various pathogenic disorders [5]. Particulate systems are considered as foreign bodies and are phagocytised by macrophages, this natural immunological response can be utilized for targeting drugs to macrophages through their entrapment into particulate systems. Microparticles for delivery of antitubercular drug through pulmonary route should provide optimum deposition to deep lungs where the alveolar macrophages reside. AntiTB drugs are always to be used in combination to avoid the chances of drug resistance. In any combination of antiTB drugs, isoniazid is always recommended as first line agent as per WHO guidelines.

Chitosan is a linear polysaccharide composed by units of glucosamine and N-acetyl-glucosamine linked by [1–4] b-glycosidic bonds. It is obtained by the alkaline deacetylation of chitin [6]. Since TB mainly attacks lungs, enhancing pulmonary anti-tuberculosis drug concentration has been one of the principal goals for improving therapeutic effect. Therefore, growing attention has been given to pulmonary route due to the fact that it allows high drug concentration to be achieved in the lesions and lung tissues and minimizes systemic side effects [7, 8] Instead of traditional oral administration route, pulmonary inhalation has been increasingly used in clinical treatment of pulmonary tuberculosis. Natural polymers present an alternative choice to synthetic polymers for various drugs delivery, including chitosan, alginate

and hyaluronic acid [9].

In biological systems, vitamin C (VC) plays the role of an effective antioxidant due to the presence of the enediol moiety. It also serves as a cofactor in hydroxylation reactions and scavenges reactive oxygen species. The biochemical functions of VC, especially its functions in antivirus and antitumor are of increasing interests [10]. However the use of VC is limited by its physical and chemical instability. VC is a six-carbon keto-lactone, which contains four hydroxyls and a lactone. It is highly unstable and very easy to get oxidized and changes to dehydroascorbic acid when exposed to light, air and elevated temperature. To increase the stability of VC, various derivatives have been synthesized, including the metal salts (Na, Ca salts), ethers, esters and the polysaccharide derivatives [11].

CS contains amino groups which are protonatable in the acidic media [12]; here we were used ascorbic acid for solubility of chitosan. Drug delivery systems that can precisely control the release rates or target drugs to a specific body site have had an enormous impact on the healthcare system. 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.

Ascorbic acid (Vit C) is a water-soluble and strong antioxidant presented in many vegetables and fruits. It is essential for collagenesis in living organism, which protect tissue and cells from oxidation reactions by free radicals and other reactive oxygen-derived species. In cosmeceutical field, ascorbic acid has been scientifically proven that it can promote the synthesis of collagen and visibly reduce the effects of skin wrinkles [13].

In biological systems, Vit C plays the role of an effective antioxidant due to the presence of the enediol moiety, that causes its mycotoxic action by driving an iron-dependent Fenton reaction that produces reactive oxygen species (ROS), which can induce cell death via DNA damage (Vilchèze *et al.* 2013). It also serves as a cofactor in hydroxylation reactions and scavenges reactive oxygen species. The biochemical functions of vitamin C, especially its functions in antivirus and antitumor are of increasing interests [14]. Intracellular location of the bacteria protects them from the host defense mechanisms and restricts the penetration of antibiotics [15]. For effective treatment of pulmonary tuberculosis, delivery system should have the ability to deliver antitubercular drug within the alveolar macrophages to eradicate the mycobacteria because alveolar macrophages serves as sanctuary for the lodging and growth of bacteria.

Our objective in present investigation is to develop microparticulate system(s) for antitubercular drug by modified ionic gelation method for inhalational delivery of drug toward alveolar macrophages to explore alternative to current available therapeutic modalities.

II Material and Methods

2.1 Materials

Rifampicin was obtained as a gift sample from Lupin Pharmaceuticals Aurangabad, India. Chitosan, a linear polysaccharide of deacetylated 1, 4-$d-$glucosamine (MW˜1,50,000 Da, $75-85\%$ Deacetylated) was acquired from Sigma Aldrich, India. Ascorbic acid and all other reagents applied in the current study were of the highest purity grade. Deionized water equivalent to Milli$-Q^{TM}$ grade was used to prepare all the solutions.

2.2 Preparation of Carrier System

2.2.1 Preparation of Stock Solution of Chitosan

First of all prepared chitosan stock solution with ascorbic acid in place of acetic acid and optimization of concentration of ascorbic acid for solubility of chitosan. To optimize the effect of concentration of ascorbic acid on solubility of chitosan solution (1%) was performed on temperature 40 ℃ and pH of final formulation was determined by pH meter. Results were shown in Table 1:

Table 1: Preparation of Stock Solution of Chitosan

Chitosan Concentration (%)	**Ascorbic Acid Concentration**	**pH of Final Solution**	**Stability/ Physical State of Solution**
1	0.05	6.9	Not Soluble
1	0.1	6.8	Very Slightly Soluble
1	0.5	6.7	Slightly Soluble
1	***1.0***	***6.7***	***Slightly Soluble***
1	1.5	6.5	Soluble
1	2.0	6.4	Soluble

2.2.2 In Situ Synthesis of chitosan microsphere

chitosan/TPP microspheres were prepared by ionotropic gelation method following a preliminary study that optimizes the preparation parameters of plain microsphere with particle suitable for pulmonary delivery (Parikh et al. 2014). Briefly, an amount of 2 ml TPP 1% w/v containing drug (2.0% w/w) was added to 5 ml of chitosan solution (1%w/v) in 1.5% ascorbic acid, under magnetic stirring at room temperature for 30 min. The dropping rate and falling distance of TPP solution were kept constant. The solution was magnetically stirred for half an hour followed by filtration and rinsing with distilled water. Then spontaneously formed microsphere suspension was then centrifuge at 5000 rpm for 5 min. The microspheres were washed two times with distilled water. The formed pellets were then freeze dried and optimized.

2.2.3 Mannosylation of Chitosan Microspheres

Mannosylation was carried out by adopting the method describe [16] with certain modification. The coupling of mannose to the amine group of chitosan was carried out in two steps. For this coupling reaction initially D-mannose (10 μm) was dissolved in 0.1 M sodium acetate buffer at pH 4 and 60°Cfor 2 hr resulting in the ring opening of mannose molecule [17].

In second step, this solution was mixed thoroughly with chitosan microsphere synthesized earlier and incubated for 24 hr at room temperature. The aldehyde group of ring opened mannose reacts with amine group of chitosan, yielding mannosylated chitosan and. The resulting solution centrifuged at 5000 rpm and washed thoroughly with distilled water. Purification of MCMs: Formulation were purified by

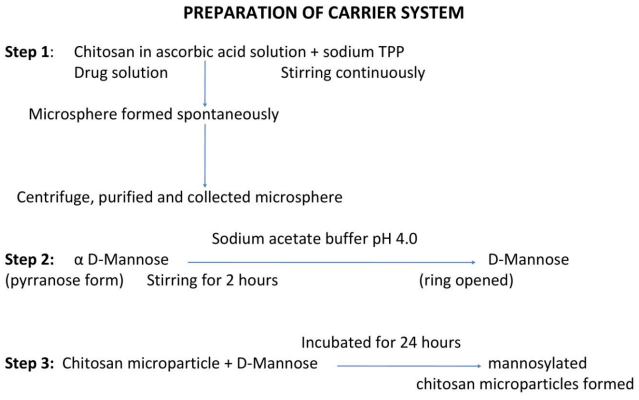

Figure 1: Schematic representation of method of preparation of carrier system

dialyzing against double distilled water in a dialysis tube (> 900KDa MWCO) for 24 hr to remove any unreacted drug and polymer.

2.3 Optimization of Formulation and Process Variables for Chitosan Microparticles

Various formulation and process variables i.e. concentration of chitosan, concentration of STPP, pH of chitosan solution pH of STPP solution, stirring speed and stirring time which affect characterization parameters of microspheres, were optimized on the basis of their effect on drug entrapment efficiency and particle size. The various variables were optimized by varying one variable at a time and keeping other variable constant.

2.3.1 Optimization of Formulation Variables

(a) **Effect of Chitosan Concentration:** Different concentrations of chitosan polymer (0.5 to 2.5% w/v) (Table 2) were used for optimizing the amount of polymer to be used in preparation of optimized microsphere. The effect of amount of chitosan on size, PDI and %EE was evaluated keeping all other factors constant. The observations are recorded in Table 3.

Table 2: Selected Factors of Formulation Variables

Formulation Code	Concentration of Chitosan (% w/v)	Concentration of STTP (% w/v)
CMAP1	0.5	5
CMAP2	1	5
CMAP3	***1.5***	***5***
CMAP4	2.0	5
CMAP5	2.5	5

Table 3: Effect of Chitosan Concentration on Average Particle Size, Zeta Potential and Polydispersity Index of CMs

Formulation Code	Average Size (μ m)	Zeta Potential	PDI
C1MAP	1.98 ± 0.78	24.2 ± 2.2	0.67 ± 0.06
C2MAP	2.40 ± 0.12	25.4 ± 3.5	0.68 ± 0.05
C3MAP	***3.58 ± 0.34***	***36.2 ± 1.2***	***0.75 ± 0.03***
C4MAP	5.66 ± 0.54	38.5 ± 3.1	0.71 ± 0.18
C5MAP	6.13 ± 0.25	39.2 ± 2.9	0.68 ± 0.20

All values are expressed as mean± S.D. ($n = 3$)

(b) **Effect of STPP Concentration:** Different concentrations of STPP (1.0 to 10 % w/v) (Table 3) were used for optimizing the % of STPP to be used in preparation of optimized microsphere. The effect of STPP on size, PDI and %EE was evaluated keeping all other factors constant. The observations are recorded in Table 4.

Table 4: Selected Factors of Formulation Variables for STPP Concentration

Formulation Code	Concentration of Chitosan (% w/v)	Concentration of STTP (% w/v)
CMAP1	1.5	1.0
CMAP2	1.5	2.5
CMAP3	***1.5***	***5.0***
CMAP4	1.5	7.5
CMAP5	1.5	10.0

(c) **Effect of pH of Chitosan Solution:** Different pH of chitosan solution (4.0 to 7.0 % w/v) (Table 6) were used for optimizing the pH of chitosan solution for interaction with STPP to be used in preparation of optimized microsphere. The effect of pH on size, PDI and %EE was evaluated keeping all other factors constant. The observations are recorded in Table 4.

Table 5: Effect of STTP Concentration on Average Particle Size, Zeta Potential and Polydispersity Index

Formulation Code	Average Size (μ m)	Zeta Potential	PDI
C3MAP1	2.10 ± 0.40	36.2 ± 2.6	0.52 ± 0.16
C3MAP2	2.48 ± 0.52	38.1 ± 3.1	0.62 ± 0.09
C3MAP3	***3.10 ± 0.20***	***39.4 ± 2.9***	***0.41 ± 0.08***
C3MAP4	4.62 ± 0.48	26.1 ± 2.4	0.78 ± 0.21
C3MAP5	4.19 ± 0.51	22.9 ± 1.2	1.02 ± 0.60

Table 6: Selected Formulation Variables for STPP Concentration

Formulation Code	Concentration of Chitosan (% w/v)	Concentration of STTP (% w/v)	pH of Chitosan Solution
C3M1AP3	1.5	5.0	4.0
C3M2AP3	1.5	5.0	5.0
C3M3AP3	1.5	5.0	6.0
C3M4AP3	***1.5***	***5.0***	***6.5***
C3M5AP3	1.5	5.0	7.0

Table 7: Effect of pH of Chitosan Solution on Average Particle Size, Zeta Potential and Polydispersity Index of CMs

Formulation Code	Average Size (μ m)	Zeta Potential	PDI
C3M1AP3	2.91 ± 0.12	38.2 ± 1.6	0.72 ± 0.06
C3M2AP3	2.98 ± 0.16	39.4 ± 2.9	0.68 ± 0.19
C3M3AP3	3.25 ± 0.20	40.6 ± 2.5	0.64 ± 0.08
C3M4AP3	***3.66 ± 0.05***	***41.6 ± 2.4***	***0.71 ± 0.11***
C3M5AP3	4.31 ± 0.14	35.5 ± 1.2	0.69 ± 0.01

(*d*) **Effect of pH of STPP Solution:** The effect of pH of STPP optimized (Table 4.9) from pH 5-9 in order to observe the effect on size, PDI and zeta potential keeping all other factors constant. The observations are recorded in Table 4.9.

Table 8: Selected Formulation Variables for STPP Concentration

Formulation Code	Concentration of Chitosan (% w/v)	Concentration of STTP (% w/v)	pH of Chitosan Solution	pH of STPP Solution
C3M1AP3	1.5	5.0	6.5	9.0
C3M2AP3	1.5	5.0	6.5	8.0
C3M3AP3	1.5	5.0	6.6	7.0
C3M4AP3	***1.5***	***5.0***	***6.5***	***6.0***
C3M5AP3	1.5	5.0	6.5	5.0

2.3.2 Optimization of Process Variables

(*a*) **Flow Rate of the TPP Solution:** The flow rate of the TPP solution was varied between 2 and 8 ml/min. By increasing the TPP solution flow rate, a small decrease in particle size and polydispersity was observed. This effect could be due to the increase in mixing where the mean diameter of the chitosan-TPP microspheres decreased when increasing the stirring speed of a magnetic stirrer. The highest flow rate 8 ml/min provided the particles with the smallest size (93 nm ±0) and polydispersity index (0.24 ± 0.00).

Table 9: Effect of pH of STPP Solution on Average Particle Size, Zeta Potential and Polydispersity Index of CMs

Formulation Code	Average Size (μ m)	Zeta Potential	PDI
C3M4A1P3	2.81 ± 0.12	28.2 ± 1.6	0.66 ± 0.06
C3M4A2P3	2.98 ± 0.16	33.2 ± 2.9	0.78 ± 0.19
C3M4A3P3	2.25 ± 0.20	35.6 ± 2.5	0.45 ± 0.08
C3M4A4P3	***3.16 ± 0.05***	***39.5 ± 2.4***	***0.65 ± 0.11***
C3M4A5P3	3.10 ± 0.14	35.5 ± 1.2	0.25 ± 0.01

(*b*) **Stirring Speed and Stirring Time:**
The stirring speed was varied between 500 and 1100 rpm, corresponding to different shear stress at the membrane surface [18]. When increasing the stirring speed, a small decrease in mean particle size and increase in polydispersity index was observed. Chitosan-TPP microspheres formation occurs through two steps: (1) formation of small primary microspheres, and (2) their higher order aggregation into larger colloids, which is mediated by TPP bridging. Faster mixing of TPP into the particle suspension reduces the extent of TPP-mediated higher order aggregation (which occurs in the presence of free TPP) by quickly reducing the local TPP concentration [19].

The stirring speed of 800 rpm was thus selected due to the optimum size (93 ± 0 nm) and polydispersity index (0.24 ± 0.00) obtained. Finally, the effect of stirring time was investigated between 15 and 45 min, and did not show any influence on the size and polydispersity of the nanoparticles. Consequently, the shorter time (15 min) was chosen for the following experiments.

Table 10: Selected Process Variables for STPP Concentration and its Effect on Average Particle Size

Parameter	mL/min	Average Size
Flow Rate of TPP (in mL/min)	2	6.2±0.91
	4	5.0±0.62
	6	4.1±0.15
	8	3.4±0.67
Stirring Speed (in rpm)	500	4.21±0.24
	800	3.98±0.41
	1100	5.42±0.35
Stirring Time (in min.)	15	3.91±0.11
	30	3.89±0.21
	45	3.95±0.14

2.4 Optimal Parameters

Table 11: Optimal Parameters

Parameter		Optimized Factor
Formulation Variables	Chitosan Concentration	1.5%
	STPP Concentration	5%
	pH of chitosan solution	6.5
	pH of STPP solution	
Process variables	Flow rate of STPP solution	8 mL/min
	Stirring speed	800 RPM
	Stirring time 15	15
	Stirring Time (in min.)	15

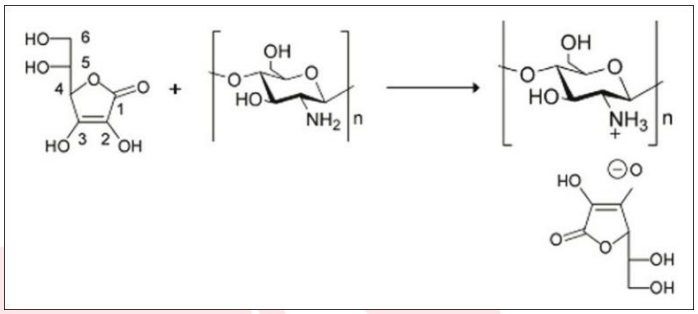

Figure 2: Interaction between Vitamin C and Chitosan Polymer during Solubilization

III Result and Discussion

There are many methods reported in literature for the preparation of chitosan microspheres for achieving efficient drug delivery. Some of the important methods are emulsion crosslinking, chemical cross-linking, coacervation, spray drying, precipitation and ionotropic gelation. Some of these methods use organic solvents and chemicals, whose residual amount in the microspheres may cause undesirable effects like irritation to mucosal membranes. Chitosan microspheres prepared by ionotropic gelation method [20] have gained importance because by this method, the microspheres could be easily prepared using simple instruments and the drug could be entrapped within chitosan microspheres in a completely aqueous environment under mild conditions.

Chitosan, a polycationic polysaccharide is insoluble in alkaline and neutral pH, but soluble in solution having acidic pH below 6.5. The amine group undergoes protonation in acidic environment that increases its solubility in acidic solution. The protonated amine group of chitosan interacts with phosphate ions provided by TPP, either by intermolecular or intramolecular linkage and the drug is entrapped within the microspheres. Here, we were used ascorbic acid for solubility of chitosan polymer to provide acidic medium.

Ascorbic acid solubilize the chitosan because chitosan contains amino groups which are protonatable in the acidic media, whereas ascorbic acid contains acidic hydroxyl functionality, thus allowing the formation of a complex through ionic interaction Figure 2. Because it plays the role of an effective antioxidant due to the presence of the enediol moiety, that causes its mycotoxic action by driving an iron-dependent Fenton reaction that produces reactive oxygen species (ROS), which can induce cell death via DNA damage [21]. It can be increase the therapeutic effectiveness of carrier system. Various optimization parameters studied and seen the effect on particle size, zeta potential and PDI. Generally, particles having particle size between $1-5$ μm penetrate deeply into the lungs and thereby reach to the alveoli. However, $1-5$ μm particles tend to agglomerate because of increased Vander waals attraction. Furthermore, cationic surface charge of the microspheres would facilitate adsorption to the alveolar cells at relatively low pH milieu in comparison to normal body pH.

As can be seen from Table 1, when increasing the concentration of chitosan, the zeta potential increased due to the increase in the positive charge of the chitosan molecules. Higher chitosan concentration caused more unneutralized - NH_3+ on the nanoparticles surface, which led to a strong electrostatic repulsion between particles [22]. This confirms that the formation of microspheres greatly depends on the concentration of free amine groups which increase the surface charge and zeta potential of the microspheres. In addition, when increasing the concentration of chitosan, the particle size increased in a linear manner with a correlation coefficient of $R^2 = 0.99$. The same result was obtained by several authors [23, 24]. Moreover, nanoparticles polydispersity increased strongly with chitosan concentration. **CMAP3** selected as optimized because the average size comes at this concentration were suitable for pulmonary delivery of drugs.

Increasing the concentration of TPP, by keeping the same concentration of chitosan, reduced the zeta potential of chitosan-TPP micropsheres. This phenomenon can be explained by the fact that at higher concentration, TPP brings more negatively charged phosphate ions which react with chitosan amino groups. Therefore, the positive surface charge of the microspheres decreases. In addition, the microspheres size increased when increasing TPP concentration. At low TPP concentrations (1%), the amount of TPP was not enough to fully crosslink chitosan chains, and the preparations remained transparent. Further increase in TPP concentration (1%) led to an increase in particle size and to a slightly opaque solution. At higher TPP concentration (5%), very large and polydispersed particles were obtained. The same result was reported already [25] who observed physical states of chitosan-TPP microspheres being either suspension (below a certain TPP concentration which depends on chitosan concentration) or precipitation (above a certain TPP concentration) in the range of TPP concentrations tested.

Chitosan is insoluble at neutral and alkaline pH. In an acid medium, the amine groups are positively charged, which gives the polysaccharide a high charge density. Here ascor-

bic acid provide acidic environment to solubilized chitosan solution. Thus, by increasing the pH of the chitosan solution, the zeta potential decreased. The ionic crosslinking process for the formation of chitosan-TPP microspheres is pH sensitive, providing the ability to modulate the composition and properties of the chitosan-TPP microspheres. From Table 1, it can also be seen that by increasing the pH of the chitosan solution, the microspheres size increased, although the polydispersity was kept almost constant. By increasing the pH, the degree of protonation at the particle surface is reduced, decreasing electrostatic repulsion between the particles thereby increasing the probability of particles aggregation [10]. Therefore, the increase in the average particles size when increasing the pH, may be mainly due to particle aggregation, rather than to the continuation of the growth of individual particles after initial formation.

Regarding the effect of TPP solution pH, shows a significant ($p<0.001$) decrease in size and polydispersity index among all formulae with decrease in TPP solution pH. Environmental pH can only be varied within a restricted range, since acidic pH will lower the charge density of TPP, decreasing its cross-linking capability, while pH larger than 6 would lower charge density of chitosan, decreasing not only its capability to cross-link but also its solubility and thus promoting its aggregation. RIF shows distinct pH dependent solubility characterized by very poor solubility in acidic conditions. Improvement of RIF % by decreasing pH of TPP solution from its original alkaline pH (8.9-9) is due to inhibition of drug loss from the microspheres due to decreased drug solubility in aqueous phase as TPP pH decreased [26].

IV Conclusion

From the results of various experiments it can be concluded that optimized factor were selected for the preparation of carrier system. From the particles size and surface characteristics, it can be concluded that microspheres of rifampicin loaded chitosan microspheres were of $1-5\ \mu$ can be prepared using ionic gelation which can be suitable for pulmonary drug delivery and provided the advantage of controlled release characteristics deep inside the lung where tubercular bacilli reside and with suitable for pulmonary drug delivery it may help in improving treatment of tuberculosis. In future prospect, the in vivo experimentation should be required to study the target potential of prepared microspheres of rifampicin.

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