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Graphical abstract

A) Endocytosis

B) Receptor internalization

- Disulfide group of Polymer + Thiol groups on membrane
- Covalent bond
- Intracellular trafficking
- Endocytosis
- MPTCh-NCs

- MPTCh-NCs
- Man
- Macrophage uptake
- Mannose receptor
- Flow uptake
- Flourescein isothiocynate NCs

- P-gp efflux
- Endosomal escape
- Mtr (Mycobion reductase level increased Reductive Environment after Mycobacterium infection)

- Intracellular trafficking
- Endosome
- RV1258
- Lysosome
- Apoptosis
- Phagosomal lysosomal fusion

- Macrophage microenvironment
Investigating the intracellular bactericidal effects of Rifampicin loaded S-protected thiomeric chitosan nanocargoes against *Mycobacterium tuberculosis*

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Abstract

The antibiotic drug resistance in *Mycobacterium tuberculosis* (M.tb) is typically associated with immune evasion shared by pathogenic bacterium and intrinsic antimycobacterial drug resistance. These factors significantly contribute to the limited delivery of drugs intracellularly thereby posing an ever-growing threat to mankind. A promising approach to tackle this multi-drug resistance is to use nanocargoes (NCs) based drug delivery approach. The aim of the present study was to develop mannose coated S-protected thiomeric site-specific nanocargoes (MPTCh-NCs) of Rifampicin (Rif) in order to deliver drug locally inside the macrophages. This NCs-based delivery system modifies the macrophage activation states via mannose receptors and endocytosis to alter the macrophage activation state thus providing synergistic antimycobacterial effects. MPTCh-NCs were synthesized by ionic gelation method and assessed for particle size and encapsulation efficiency. Moreover, MPTCh-NCs were also investigated in *in vitro* for drug release, macrophage uptake, buffering potential, Mycothione reductase (MTR) inhibition ability, inhibitor concentration (MIC), phagolysosomal fusion, reactive oxygen species (ROS) production apoptosis and RV 1258 inhibition. The *in vivo* bioavailability study of MPTCh-NCs was also evaluated in male BALB/c models over a period of 72 h. The optimized MPTCh-NC formulation was nanosized (390 ± 20 nm) with better EE of Rif i.e. 73.68 ± 5.99 %. The MPTCh-NCs showed better buffering capacity at different pH ranges, 35.69 folds higher macrophage uptake than Rif with P-gp inhibition potential and pronounced MTR inhibition potential. The MPTCh-NCs exhibited MIC of 16 µg/ml by drug susceptibility testing. Flow cytometric analysis of MPTCh-NCs exhibited, increased apoptosis (33.29 %). Real time PCR data suggested enhanced RV 1258 inhibition potential (0.387 fold expression) of the MPTCh-NCs. *In vivo* results indicated increased bioavailability of MPTCh-NCs (AUC 12.31 folds higher) in comparison to conventional drug Rif. In summary, the observed capacity of the mannose coated S-protected NCs-based approach to deliver therapeutic levels of Rif selectively has potential to improve the therapeutic management against drug resistant tuberculosis.

**Key words:** multidrug resistance, nanocargoes, anti oxidant potential, Mycothione reductase, alternative activation state, S-protected chitosan
1. Introduction

Tuberculosis (TB) is the deadliest infectious disease with two million deaths annually across the globe. Despite efforts to mitigate its toll on humanity, it is still considered a major threat to public health. The microenvironment conditions and lesions of TB are highly complex and induce diverging trajectories of these lesions within the host’s innate immune system. The microenvironment complexity is due to the multiple types of lesions associated with *Mycobacterium tuberculosis* (M.tb). TB microenvironment not only initiates a plethora of immune evasion but also in parallel, provides a niche for *Mycobacterium* survival [1, 2]. The host’s immune system limits the mycobacterial spread through a series of events induced by immune system. One of the major limitations of the existing therapeutic modalities is the intra-macrophage localization of the *Mycobacterium*. The p-glycoprotein efflux pumps (EPs) existing on the surface of macrophages is involved in the rapid efflux of certain drugs such as Rifampicin, moxifloxacin, isoniazid and bedaquiline [3]. Additionally, endosomal encapsulation of the drug results both in drug deterioration [4] and in phagolysosomal fusion [5]. The ability of *Mycobacterium* to circumvent the macrophage induced ROS depends on the reduction of mycothiol (MSH). . Enzymes involved in the MSH biosynthesis are considered to be essential for the growth of M.tb. Mycothione Reductase (MTR) is one of these enzymes, which actively participates in M.tb growth. MSH is being converted into its oxidized form such as MSSM as a result of its exposure to ROS. In order to maintain this reducing potential, MTR also catalyzes MSSM and forms MSH. Moreover, MSH plays a pivotal role in *Mycobacterium* survival[6].

*Mycobacterium* has EPs existing on its surface which are actively involved in the expulsion of drugs (such as RV1258 for Rif) which in turn diminishes the intracellular drug concentrations. Such strategies mediated by *Mycobacterium* facilitate the reduced intracellular drug levels and subversion of immune response thereby producing the alternative pathways for the activation of macrophages [3, 7].

There is an urgent need to develop a drug delivery platform that addresses aforementioned challenges to improve intracellular trafficking, subvert the immune response and to facilitate synergistic bactericidal effects. Different studies have shown that the alternative activation state of macrophages results in the deprivation of coordinated defense systems of M.tb [8]. Nanotechnology has gained much attention owing to their tunable shape and size-dependent
physiochemical features. NP-based cargo systems (known as nanocargoes) advance the therapeutic and pharmacological performance of chemotherapeutic agents via targeted delivery and controlled drug release. This targeted and controlled drug release eliminate the problems associated with conventional drugs such as non-selectivity and uncontrolled/unpredictable release [9]. Several nanotechnology-based drug delivery strategies have been developed in order to obtain macrophage activation. For instance, Pi et al., reported that the phagocytosis of the Selenium-nanoparticles (NPs) increased the phagolysosomal destruction of macrophages and enhanced the ROS-mediated programmed cell death [10]. In another study, it has been demonstrated that the phagocytic uptake of glucan-NPs of Rifabutin resulted in the activation of cascade responses within infected macrophages. These responses included enhanced ROS generation, apoptosis and phagolysosomal destruction of the Mycobacterium [5]. Therefore, NPs-based delivery formats have emerged as a promising candidate in drug delivery applications. In these regards, a nanocargo (NCs) is a promising solution owing to their high abilities of carrying drugs and releases them selectively (where the drug release is the most needed). Successful delivery of NCs into the cytoplasm of cells involve three critical steps: 1) cellular internalization and localization; 2) stimulation of endocytosis and 3) facilitation of endosomal escape [11]. S-protected thiolated polymers (also known as thiomers comprising thiol groups covalently attached to their backbones) have widely been studied for their increased cellular uptake, efflux pump, enzyme inhibition and permeation enhancing features [12].

In this work, we developed a smart, site-specific, S-protected thiomeric chitosan NCs coated with mannose in order to increase the intracellular drug trafficking via two ways. Firstly this thiomeric drug delivery platform containing disulfide bonds at the surface of the polymer enables NCs to pass through the cell membrane by endocytosis entry pathways [11]. Secondly, mannose receptor internalization facilitates the carrier system (MPTCh-NCs) to pass through the macrophage. As prepared NCs were characterized to investigate their increased intracellular drug uptake, P-gp EP inhibition, endosomal escape potential, MTR inhibition and enhanced reactive nitrogen species (RNS) & ROS-mediated cell deaths. The MPTCh-NCs were also evaluated for enhanced phagolysosomal fusion, decreased MIC, inhibition of RV1258 EP and increased bioavailability in vivo. Thus we report the mannose coated S-protected thiolated nanocarriers (MPTCh-NCs) with enhanced drug targeting intracellularly while altering the immune escape.
strategies of *Mycobacterium* thus increasing the pathogen insult via synergistic mycobacterial effect.

2. Materials and Methods

2.1. Chemicals and reagents

Chitosan (50,000 Da) with the degree of deacetylation 75-85%, mercapto nicotinic acid, sodium tripolyphosphate (TPP), thioglycolic acid (TGA) and D-mannose were purchased from Sigma-Aldrich, Germany. Hydroxylamine, 1-ethyl-3-(3-dimethyl aminopropyl carbodiimide hydrochloride (EDAC), Ellman’s reagent and sodium *cyanoborohydride* were purchased from Merck, Germany. Ascorbic acid and dimethyl sulfoxide (DMSO) were purchased from Merck, Pakistan. Penicillin, Streptomycin and RPMI were purchased from Merck, Pakistan. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Merck, Germany. Rifampicin was given as gift form Pfizer Laboratories Ltd. All solvents used were of HPLC and analytical grade. All the chemical reagents and solutions were used without any further modifications.

2.2. Synthesis of polymeric carrier and basic characterization of nanocargoes

The detailed methodology of synthesis of thiolated chitosan, quantification of thiol, mercapto nicotinic acid, mannose and disulfide linkage, H$^1$NMR (nuclear magnetic resonance), fourier transform infrared (FTIR), differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) are given in supplementary information section.

2.2.1. Formulation optimization by design expert

For the optimized synthesis of nanocargoes Design Expert® software was utilized to optimize the ratio of different ingredients i.e. conjugated polymers (Ch, TCh, PTCh and MPTCh), and tripolyphosphate (TPP) using the central composite design (CCD) The optimization was carried out in terms of particles size, zeta potential, polydispersity index (PDI) and entrapment efficacy (EE) as dependent variables. The matrix generated by the software was used to prepare the actual formulations and analyzed statistically, including linear regression and response surface analysis. Data with p-value < 0.05 was considered significant and included in the model. Based on better fit (including probability F-value, adjusted R-square, noise level, adequate precision and lack of fit F value), the best mathematical model was chosen for each response.
NCs were formulated by ionotropic gelation as reported previously, using the optimized ratios suggested by the Design expert® [13, 14]. Blank NCs were prepared by dissolving the polymer (0.2%) in deionized water and TPP (0.2%) was added dropwise until the appearance of opaque color. For the preparation of enveloped nanocargoes, 0.2% solution of conjugated polymers (Chitosan=Ch, thiolated chitosan=TCh, S-protected thiolated chitosan=PTCh, and mannose-grafted S-protected thiolated chitosan=MPTCh) in 1% (v/v) acetic acid (pH 4.0), and 0.2% TPP solution in deionized water was prepared separately. The specified amount (1mg/ml) of Rifampicin (Rif) was dissolved in DMSO and diluted with the PBS (pH 4.0). The Rif solution was added to the TPP solution and added dropwise to the polymer solution until the appearance of translucent color. The resultant suspension was centrifuged at 13,500 rpm for 30 min and the pellet of NC was collected and re-dispersed in 3% trehalose solution, freeze-dried and stored at 4°C [13, 14].

2.2.2. Basic characterization of NCs

Drug-loaded nanocargoes and blank nanocargoes were analyzed for particle size, zeta potential and polydispersity index (PDI) using Nanozeta sizer (Malvern, UK) by diluting the samples 10 times so that the electrophoretic mobility of nanocargoes should not be compromised by the aggregation [15].

The surface morphology was analyzed using scanning electron microscopy (SEM) (FEI Nova NanoSEM 450, USA). Samples for SEM images were carefully prepared by slow evaporation of a single dilute drop of formulation on carbon-coated copper grid followed by blotting with a drop of 1% ammonium molybdate solution. For better contrast, the dried sample was further coated with gold, using sputter coater (Denton, Desk V HP) operating at 40 mA for 15 sec under vacuum [14].

The encapsulation efficiency (EE) of Rif was measured by the indirect method [16]. The suspensions of prepared nanocargoes were centrifuged (14,000 g) for 15 min and supernatant was collected [17]. The supernatant was analyzed, using the HPLC method described above, for the quantification of Rif. The encapsulation efficiency was calculated by the formula:
2.2.3. **In vitro drug release**

In vitro drug release profile of the polymeric (Ch, TCh, PTCh, and MPTCh) NC was studied at physiological pH i.e. 7.4 (PBS) and macrophage endosomal pH 5.5 (PBS) by using the dialysis tube technique. Briefly, NC suspension equivalent to 10 mg of Rif was taken in the dialysis tube (MW cut off =12-14 kDa) and suspended in the beaker containing the dissolution medium of 50ml at 37 ± 2°C along with 0.1 mg/ml of ascorbic acid to prevent the oxidation of Rif. Sink conditions were maintained by adding 1% tween in PBS which allows the total quantity of drug to be eluted from nanocarrier and reduced adhesion by using dialyzing membrane. The sample was set at 50 rpm and at specific time intervals (1, 2, 4, 8, 12, 24, 48, 72 h), the samples were withdrawn and replaced with fresh medium of the same volume and drug content determined through HPLC method described in section electronic supplementary information [18].

2.3 Detection of buffering potential of the polymeric carriers

Acid base titration was used in order to evaluate the buffering potential of Chitosan (Ch), Thiolated chitosan (TCh), S-protected thiolated chitosan (PTCh) and Mannosylated S-protected thiolated chitosan (MPTCh). Briefly, the polymeric NCs were dissolved in 0.1N NaCl at a concentration of 0.1mg/ml with pH adjustment of 10. Afterwards, 0.1 M HCl was added dropwise (20 µl) into the polymer solution while different pH values were measured by using pH meter. The slope of the plot between pH and HCl amount indicated the buffering capacity of our polymeric system [4].

2.4. Biocompatibility, macrophage uptake, mycobacterial inhibition potential and P-gp inhibition studies

2.4.1 **Bacterial culture**

*Mycobacterium* strains (H37Rv and R-1343) were obtained from National Reference Laboratory, National Institute of Health, Islamabad, Pakistan. The strains were grown in the mycobacterium growth indicator tube (MGIT 960) added with OADC (oleic acid, albumin, dextrose and catalase) along with antibiotic combination (PANTA) to inhibit the growth of any other bacterial strain and to prevent contamination.
2.4.2 Macrophage isolation and infection

Macrophages were isolated from female swiss albino mice using previously reported method [19]. All the animal experiments were approved by the local ethical committee i.e. from bioethical committee Quaid-i-Azam University, Islamabad (BEC-FBS-QAU2019-202) which are approved according to the ARRIVE guidelines and the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. The animals of 20-25 ± 5 g were kept under 12 h light and 12 h dark cycles with free access to food and water. Animals were acclimatized for 3-4 weeks and their body weights were measured in order to evaluate the health of the animals and to ensure if they were in an excellent state to perform any experimentation upon them. The animals were used in minimum possible number and the methods applied give minimum stress to animals. Also the sacrifice of animals was done to give the less possible pain of death. Briefly, 1.5 ml of sterile thioglycolate (3% w/v) was inoculated into the peritoneal cavity of swiss albino mice. After 3 days the mice were euthanized and ice cold RPMI (5ml) was injected into the peritoneal cavity of mice. The peritoneal exudate was then collected and recovered. The exudate collected above was then processed further followed by the centrifugation of 10 min at 3000 rpm. The pellet recovered was then suspended in RPMI supplemented with penicillin, streptomycin and 10% FBS.

2.4.3 Biocompatibility analysis and phagocytic uptake

The detailed methodology of biocompatibility and uptake (phagocytic uptake, fluorescent uptake and uptake via flow cytometry) are given in supplementary information.

2.4.4 Treatment with nanoformulations, % inhibition and drug influx by P-gp inhibition

Macrophage infection

Macrophages isolated above were plated on 24-well plate at the density of 1×10^5 cells/well with microscopic slides and incubated at 37°C in CO₂ incubator for 24h to attach the monolayers to the slides. After incubation, cells were washed with the serum free medium and adherent cells were infected with the M.tb at a ratio of 10:1 (Mycobacteria: macrophages) and then incubated for 7 days in a CO₂ incubator at 37°C. Afterward, the cells were washed with the RPMI to remove the un-phagocytosed Mycobacteria and then incubated with different concentrations of Rif and NC (1-200 µg/ml) for 24 h to examine the bacteria within infected macrophages an acid-
fast bacilli (AFB) stain kit (BD Biosciences, country) was used and staining was performed 
according to the manufacturer's instructions. The slides were then stained with the Giemsa staining 
solution for 10 min. The stained cells were then visualized under the light microscope to count 100 
cells per well to compute the percentage of infected macrophages per 100 cells. Percentage of 
inhibition was calculated by the following equation [20].

\[
\% \text{ inhibition} = \frac{No. \text{ of mycobacteria in control well} - No. \text{ f mycobacteria in treated well}}{No. \text{ of mycobacteria in control well}} \times 100
\]

In order to evaluate the drug uptake of NC by P-gp efflux pump inhibition, the macrophages 
isolated above (1×10^5 cells/well) and infected with M.tb were treated with adenosine triphosphate 
binding cassette (ABC) inhibitor i.e. verapamil (5 μM) before adding the NC (at their MICs). 
Verapamil was added in resistant strain (R1343) infected macrophages, sensitive strain (H37Rv) 
infected macrophages and non-infected macrophages. After treatment of 2 h, cells were washed 
with buffer (pH 7.4) and treated with Rif and nanocargoes. After 24 h the cells were processed 
for the HPLC analysis of drug [20].

2.5. Detection of phagosomal maturation and acidification 

The macrophages cells were isolated from the peritoneal cavity as described above. The 
macrophages (1×10^5 cells/well) infected with M.tb were exposed to the prepared NCs i.e. 
MPTCh-NCs (1 μg/ml) for 24 h and then incubated with acredin orange (AO) for 15-20 min in 
order to detect the acidic vesicular organelles. The acidic compartments fluorescence red color 
(488 nm) while nucleolus and cytoplasm fluorescence green (520 nm) and this intensity depends 
on the degree of acidification. The fluorescent microscope (20X) was used to obtain the 
photomicrographs. To detect the phagosomal acidification upon lysosomal fusion, the treated 
cells were also stained with Lysotracker-G. After 24 h treatment with nanocargoes, the cells were 
then washed with PBS and stained with Lysotracker and further incubated at 37^0 C for 15 min. 
Afterwards the cells were washed with PBS and photomicrographs taken via fluorescent 
microscope [5, 9].

2.6. Mycothione reductase (MTR) inhibition studies 

The MTR inhibition studies of S-protected thiomeric NCs on M.tb was conducted as reported 
previously. Briefly, the crude enzyme (MTR) was prepared by incubating the mycobacterium
with lysis buffer (HEPES (40mM), triton-x 100 (2 % v/v), tris (pH 7.5, 50 mM), EDTA (1mM)) and phenylmethanesulfonyl fluoride (1mM) was added as protease inhibitor. Total of 75 µl of lysate was added into the 96 well plates along with the addition of 25 µl Ellman’s reagent (100 µm) and 25 µl NADPH (200 µm) and MPTCh-NCs at different concentrations (0-10 µg/ml). The reaction mixture was then incubated for 3 h in dark at room temperature. After incubation the absorbance was measured at 405 nm using the microwell plate reader [21]. For each sample the control was set by adding all reagents except substrate. To calculate MTR activity following formula was used:

\[
MTR = \text{Optical density of sample at 405 nm} - \text{Optical density of control at 405 nm}
\]

Different kinetic models were applied by using Graphpad Prism (model etc) and the \(R^2\) values were subjected to sum of square F-test analysis for best fit values. The IC50 value for MPTCh-NC was calculated by using graph pad prism.

2.7. Detection of oxidative burst, nitric oxide and cytokines quantification

In order to appraise the oxidative burst inside the macrophage cells induced after the uptake of the MPTCh-NC, the macrophages (1x10^5 cells/well) were incubated for 30 min with 2’, 7’-dichloroflorescein diacetate (DCFD) which is a cell permeable fluorogenic dye. Afterwards, the cells were then treated with MPTCh-NCs at a concentration of 1 µg/ml. The plate was then kept in dark on a shaker for 10 min and the fluorescence fervency was measured at 485 nm (excitation wavelength) and 530 nm (emission wavelength) by using fluorescent microplate reader [22].

The details of the nitric oxide and cytokines quantification are given in supplementary information section.

2.8. Cell annihilation analysis

The cell annihilation after the predisposition of the cells to MPTCh-NCs was evaluated by annexin-V PI staining and then quantitatively assessed by using flow cytometry. Macrophages (1 x 10^5 cells/well) were seeded in 6 well-plates, infected with M.tb and further incubated for 24 h after treatment with pure drug and NCs (Ch, TCh, PTCh and MPTCh) at a concentration of 1 µg/ml. Afterwards, the cells washed with PBS and fixed with ice cold methanol (-20° C).

Subsequently, the fixed cells were embraced with RNase and stained with annexin-V PI and kept
in dark for 30 min. Afterwards the fluorescence was measured by flow cytometer for each individual nucleus [5].

The nuclear morphological changes provoked by the treatment of NC was enumerated after 4’, 6’- diamidino-2-phenylindole (DAPI) staining which is a nuclear stain dye. The infected cells were seeded (0.2 × 10⁶ cells/well) in a 6 well-plate and treated with MPTCh-NCs (1 µg/ml) and further incubated for 24 h. Subsequently, the cells were fixed in 4 % paraformaldehyde after washing with PBS. Afterwards, the cells were lysed with lysis buffer (triton X-100) and incubated with DAPI (0.5 µg/ml) for 5 min and visualized under fluorescence microscope [23, 24].

2.9. Drug susceptibility testing and *Mycobacterial* EP inhibition

Drug susceptibility testing (DST) and *mycobacterial* efflux pump inhibition potential of the prepared NC was carried out by using growth indicator tubes, MGIT-960 based method (TB Alliance protocol NC-005-(J-M-Pa-Z)) against M.tb and MIC was determined. MGIT growth indicator tube with BACTEC MGIT-960 supplement was used for the *Mycobacterium* in the instrument. The standard protocol was carried out for all the primary drugs. The culture used for inoculation was dispersed to avoid clumps and to allow even distribution of the microbe. After 15 min these dispersed cultures were used to inoculate the MGIT tubes containing first-line drugs and ODAC (oleic acid, dextrose, albumin and catalase) supplement. The tubes were then loaded in the MGIT-960 instrument. When GU reaches 400 the instrument reads the set of DST as complete. The complete set was then removed from the instrument and print of the removed DST set was taken an interpreted manually. In comparison to control (GU = 400), if drug-containing tubes showed GU of greater than 100, the result was interpreted as resistant. While, if GU is equal to or less than 100, the result will be interpreted as susceptible. The above experiment was repeated for Rif + Ver and NC (Ch, TCh, PTCh and MPTCh) with different concentrations (0.65-100 µg/ml) in order to estimate the minimum inhibitory concentration (MIC) and EP inhibition potential [7, 25]. The modulation factor (MF) was also calculated by the following formula:

\[
MF = \frac{\text{MIC of Rif/NCs}}{\text{MIC of Rif + Verapamil}}
\]
2.10. RV1258 inhibition potential by RT-PCR and in vivo bioavailability studies

For RV1258 inhibition potential of prepared mannose coated S-protected thiolated NC of Rifampicin, the standard protocol of RT-PCR was used with slight modifications [7].

*Mycobacterial* cultures (drug sensitive and drug resistant) were obtained from national reference laboratory, NIH Islamabad Pakistan. The strains were incubated at 37°C with Rif and MPTCh-NCs at their minimum inhibitory concentrations in 7H9 broth containing ODAC (oleic acid, dextrose, albumin and catalase) medium.

RNA extraction from the *Mycobacterial* cultures grown at OD of 600 nm of 0.5 and 0.8 was done by using triazole method. Briefly, the culture was centrifuged at 4000 rpm for 20 min at 25°C and the pellet was resuspended in 1ml triazole reagent (Invitrogen, USA) in order to lyse the *Mycobacterial* culture and incubated at room temperature for 5 min. Afterwards 400µl of chloroform was added and further incubated for 3 min. The homogenate was then centrifuged at 12000 rpm at 4°C for 10 min for phase separation. Upper aqueous layer was then separated and isopropanol was added in equal ratio. The tubes were then incubated on ice (-20°C) for 10 min to precipitate down the RNA. The sample was then centrifuged at 12000 rpm and 4°C for 10 min and supernatant was discarded. The pellet was dried in the air and afterwards, 40 µl of RNAse free water was added. RNA can be stored at -80°C until further use. The quality and quantity of RNA was assessed by using Nanodrop plate (Skanit RE 4.1, Thermoscientific). Absorbances were measured at 260, 280 and 320 nm.

RNA isolated above was then reverse transcribed into cDNA by using cDNA synthesis kit (Vivantis cDSK 01-050) and quantitative real time PCR conducted by using 2X HOT SYBR Green qPCR mix (Solar Bio Cat. No. SR1110). The primers used for RV1258 and housekeeping gene are (RV1258c_F: GGGCGGGGATGCGGTTCATCGAT, RV1258c_R: ATGCGGCAACGTCGCGCATCAAG, DNAPolA_F: TCGATTGCCGGTCTTTAC, DNAPolA_R: CACCACGGCTCACACTTTAT). Real time PCR was performed on Mic PCR (Bio Molecular system) and the expression levels were then normalized to the expression level of the reference gene RV1258 [7].

Male BALB/C mice were used for in vivo analysis. All the animal experiments were approved by the local ethical committee i.e. from bioethical committee Quaid-i-Azam University, Islamabad.
The details have also been given in the previous section (2.4). The mice (20-25g) were fasted overnight with free access to water and were randomly distributed into four groups (n = 4). Rifampicin loaded NC such as MPTCh and marketed product of Rifampicin at a dose of 12 mg/kg body weight were administered to the mice by oral gavage. Blood samples (approximately 1 ml) were collected from the tail vein at different interval of 1, 4, 6, 8, 10, 12, 24, 48 and 72 h in microcentrifuge tubes containing heparin. These blood samples were centrifuged at 3500 r.p.m. for 10 min to separate the plasma, and the plasma samples were then subjected to HPLC analysis [26]. The validated HPLC method was employed as described in supplementary information.

2.11. Statistical analysis

The measurement of the significance of results was carried out by using one-way ANOVA following Tukey’s post-hoc test and student t-test where applicable. The $P < 0.05$ was considered as significant. All the results were expressed as the mean ± standard deviation of at least three (n=3) experiments.

3. Results and discussion

3.1. Synthesis, basic characterization and buffering potential of functionalized polymeric nanocargoes

Detailed synthesis and basic characterization of Ch, TCh, PTCh and MPTCh polymers have been presented in supplementary information. The structure of the final product, MPTCh is shown in Figure 1. A schematic representation of preparation of MPTCh is give in Figure S1. The results of $^1$H NMR and FTIR analysis are shown in Figure S2 and Figure S3. DSC, TGA and XRD (Figure S4) are provided in supplementary information section. Briefly, the presence of proton peaks (7-9 ppm) in the 1H NMR spectrum of S-protected thiolated chitosan are associated with aromatic ring of 6-mercapto nicotinic acid (MNA) and presence of methylene peaks (2.7 ppm and 3.5ppm ) in spectrum of mannosylated S-protected thiolated chitosan confirmed attachment of sugar moiety to the polymer. The presence of amide, aromatic and hydroxyl bands in FTIR spectra of MPTCh confirms the association of mercaptonicotinic acid and mannose sugar to the polymeric backbone. The DSC analysis of PTCh showed an endothermic peak at 240° and MPTCh showed an endothermic peak at 260° with no crystalline melting peak. XRD pattern
showed somewhat crystalline nature of all the functionalized polymers. The crystalline peaks in case of MPTCh-NCs was destroyed which suggests the greatest disarray of the polymer network by the cross linking of polymer with the TPP so the XRD pattern of MPTCh-NCs suggests somewhat amorphous nature of the nanocargoes.

Characterization of polymers (in terms of thiol contents, disulfide bonds, amount of S-protected ligand and mannose groups) is given in Table S1. Nanoformulations were prepared using the optimized ratios obtained through Design Expert® presented in supplementary material Table S2. The third formulation block suggested by Design Expert® was selected for the optimized ratios to prepare the nanoformulations. The results of the hydrodynamic diameter, zeta potential, encapsulation efficiency (EE) and polydispersity index (PDI) for Ch (chitosan), TCh (thiolated chitosan), PTCh (s-protected thiolated chitosan), and MPTCh-NCs (mannosylated s-protected thiolated chitosan) are presented in Table 1 and Figure 2A (i-iv). Based on the impact of particle size, PDI and EE one point at the optimal area was selected at which the particle size was reduced with maximum EE and suitable PDI ($p < 0.5$). Free amino groups at the polymeric surface was involved in cross linking of carrier with TPP and resulted in the spherical shaped NCs [28]. Ch-NC exhibited the maximum value ($36.3 \pm 2$) of zeta potential while the MPTCh-NC showed a minimum value ($18.4 \pm 5.46$) of zeta potential. This minimum value of zeta potential is due to the modification of free amino groups of chitosan with thiol and mannose groups. Also, the positive zeta potential of NCs is favorable for their internalization into cells, given that the electrical potential of the cell membrane is negatively charged. S-protected thiomeric chitosan coating provides the steric shielding of NCs from precipitation and aggregation by the proteins in the physiological media. These results indicated the robust stability of nanocarriers which assures the longer circulation time and more chances of macrophage accumulation resulting in enhanced therapeutic efficacy [27].

The SEM images of blank MPTCh-NCs and Rif loaded MPTCh-NCs are shown in Figure 2B, which indicated a smooth surface of the spherical particles.

Drug release from biodegradable polymeric systems follows diffusion (through water filled pores or the matrix following osmotic gradient), erosion (both surface and bulk erosion) and degradation (chemical and enzymatic degradation) [28].
One of the dominant cellular uptake mechanisms is endocytosis. This pathway consists of endosomal vesicles which grow in the form of early and late endosomes before fusion with lysosomes and contains degradative enzymes. This results in limited delivery of drugs and macromolecules to intracellular targeted sites. Several attempts have been made to directly deliver the drugs inside the cytosol thus escaping the endocytic pathway [29]. The endosomal escape potential of NCs depends on the inbred buffering capacity of the polymeric carrier. The quantity of the HCl imperative to bring the pH decrement from 10 to 2.6 increased in the order NaCl > TCh > Ch > PTCh > MPTCh confirming the accentuated buffering potential of MPTCh because of –NH2 groups of preactivation. (Figure 3A). The results of pure drug suspension showed maximum release (> 80%) in the first 4 h. Rif release from NCs (Figure 3 B, C) followed a biphasic release pattern with initial burst release of drug close to particle surface or near the water layer followed by sustained release due to slow diffusion. In general, compared to a pure drug suspension, NCs showed pH-dependent release with initial burst release (~ 30%) within first 10 h followed by a sustained release for up to 72 h. Notably, TCh-NCs and PTCh-NCs showed better control than pure drug (Rif) and Ch-NCs, releasing 80% drug after 72 h. Generally, chitosan shows a pH-dependent drug release i.e. better drug release at lower pH (pH 1-2). This might be attributed to the presence of a more complex and stronger matrix system in the order TCh > PTCh > MPTCh, providing better control over drug release. Rif release kinetics from NC was evaluated by employing various release models for both pH 7.4 and 5.5 release profiles and the results are presented in Table 2. Based on the value of R², the drug release from Ch-NCs followed first order release behavior at both pH 5.5 and 7.4 and the Rif release from TCh-NCs, PTCh-NCs and MPTCh-NCs followed the Korsmeyer-Peppas model. At pH 7.4 the release followed non-Fickian mechanism as the value of 0.45 < n < 0.89, that supported relatively larger drug release as shown in Figure 3B. The diffusional exponent (n) of Korsmeyer-Peppas model showed the release profile of TCh-NCs, PTCh-NCs and MPTCh-NCs followed Fickian at pH 5.5 due to the value of n < 0.45, which is evident in Figure 3C showing less drug release.

3.2 Biocompatibility, uptake, Mycobacterial inhibition and P-gp inhibition analysis

Biocompatibility assessment is an important parameter to study the response of living systems towards NC following administration in time and dose-dependent manners. Though chitosan and
its derivatives have been shown as less toxic and more biocompatible, yet NCs may induce some acute or moderate levels of toxicity based on their extremely small size. These toxic effects could be minimized or avoided by surface modification or tuning their properties [30]. The antimycobacterial activity of NC was investigated against M.tb in a concentration-dependent manner. Free Rif was used as a control. As shown in Figure 4A. The Rif, Ch, TCh, PTCh and MPTCh-NCs showed 40.9 ± 2%, 60.34 ± 1%, 71.98 ± 6%, 80 ± 5% and 88.09 ± 4% growth inhibition respectively. The measured IC$_{50}$ of Rif, Ch, TCh, PTCh, and MPTCh-NCs was found to be 8.71 ± 0.4, 5.82 ± 0.5, 4.55 ± 0.3, 4.04 ± 0.1 and 3.18 ± 0.5 µg/ml respectively (Figure 4B).

The biocompatibility of as-prepared NC was evaluated on fresh albino mice peritoneal macrophages using MTT assay. The macrophages were incubated with various concentrations of NCs i.e. 1-200 µg/ml. The results (Figure 4C) showed significant ($p < 0.05$) differences among various treatment groups. The results of MPTCh-NCs showed > 89 % ± 2 cell survival over 24 h at the highest concentration tested (200 µg/ml), while Ch-NCs, TCh-NCs, and PTCh-NCs showed cell survival of 67 ± 7, 81 ± 10% and 83 ± 5% respectively at the concentration of 1-200 µg/ml. Similarly, MPTCh-NCs and PTCh-NCs showed significantly ($p < 0.05$) low IC$_{50}$ of 3.6 µg/ml and 4.04 µg/ml against macrophages as compared to TCh-NCs and Ch-NCs indicating a lower toxicity of the developed MPTCh-NCs. The values for negative control (Triton X-100, 2% v/v) and positive control (RPMI media) showed 5 ± 1% and 99 ± 2% viability respectively. Improved biocompatibility of PTCh-NCs and MPTCh-NCs as compared to the chitosan is attributed to the positive charge density of the chitosan that might have interacted with negatively charged cell membrane.

Macrophage surfaces harbor mannose receptors that are over expressed in infected macrophages. PTCh and MPTCh showed increased uptake in both uninfected and H37Rv infected macrophages compared to Rif. This enhanced trafficking inside the macrophages may be attributed due to mannose receptors endocytosis as well as S-S/S-H exchange reaction of S-protected thiomer with the cell membrane [31]. Different studies provide the evidence of effectiveness of thiol moieties present on NC surface in increased internalization. The cell surface thiols further enhance the intracellular uptake of disulfide conjugated thiomic polymer (PTCh).
The fluorescent microscope images of FITC-MPTCh-NCs and macrophages with successful internalization of FITC-MPTCh-NCs are shown in Figure S 5 (A-B). The green fluorescence exhibited by macrophages confirmed the presence of FITC-MPTCh-NCs inside the macrophages. The increased fluorescence inside macrophages was because of the successful disulfide mediated endocytosis as well as mannose receptor-ligand conjugation which showed successful internalization of NCs as a whole, showing the stability of formulation until it reaches the intracellular microenvironment.

One of the resistance mechanism associated with TB drugs is the presence of efflux transporters present on macrophages as well as M.tb surface [3]. The macrophage uptake of Rif, Ch, TCh, PTCh, and MPTCh-NCs were evaluated both in infected (sensitive and resistant strains) macrophages and uninfected macrophages in the presence of verapamil to evaluate the P-gp inhibition potential. The results presented in Table 3 showed that Rif exhibited significantly reduced uptake ($P < 0.05$) in macrophages infected with resistant strains. In the case of Ch-NCs and PTCh-NCs the sensitive strain infected macrophages exhibited an uptake of $10.87 \pm 0.25 \mu g$ Rif/10$^6$ cells and $34.89 \pm 0.24 \mu g$ Rif/10$^6$ cells respectively. It is worth noticing that in case of the resistant strain infected macrophages, PTCh-NCs and MPTCh-NCs exhibited an uptake of $35.24 \pm 0.01 \mu g$ Rif/10$^6$ cells and $76.61 \pm 0.05$ that is almost equal ($P > 0.05$) to that of sensitive strain infected macrophages i.e. $34.89 \pm 0.24 \mu g$ Rif/10$^6$ cells and $76.88 \pm 0.02 \mu g$ Rif/10$^6$ cells, respectively. These results indicated that thiolated NC have successfully inhibited the P-gp EP by developing a disulfide linkage with their cystine subunit [32]. MPTCh-NCs exhibited maximum intracellular accumulation of Rif in all cases (non-infected, sensitive strain infected and resistant strain infected macrophages) compared to that of other NCs due to the macrophage-targeted potential of MPTCh-NCs. Hence, these NC might prove to be a suitable strategy to enhance the intracellular accumulation by blocking these EPs [33].

### 3.3 Phagosomal lysosomal maturation and acidification

M.tb has emerged as an innovative strategy to circumvent the phagolysosomal degradation via knockdown of phagosomal maturation processes. We hypothesize that the uptake of MPTCh-NCs could potentially enhance the lysosome accumulation resulting in an increased phagosomal maturation within infected macrophages. The accrual of green fluorescence fervency after MPTCh-NCs treatment indicated the phagosomal maturation and lysosomal buildup as depicted
in Figure S 5 (C-D). Carrier system developed in the present work releases the phagosomal maturation block and enhances the Mycobacterial insult. Based on these results, it can be concluded that MPTCh-NCs could play a vital role in autophagy within *Mycobacterium* infected macrophages, although the mechanism has not been illustrated yet. Future studies should emphasize on the exploring this mechanism.

### 3.4 Mycothione reductase (MTR) inhibition assay

The maintenance of redox balance is crucial for the survival of *M. tb* inside the host. To neutralize the oxidative stress of host immune response system, *M. tb* utilizes the unique protecting enzyme mycothione-reductase (MTR). This NADPH dependent MTR protein ensures the reductive environment by maintaining the mycothiol (MSH) in its reduced form. The inhibition of MTR by thiomers can increase the efficacy of anti-tubercular drugs. The MTR inhibitory activity of MPTCh-NP was evaluated at different concentrations (1-100 µg/ml) against 1 µm concentration of Mycothiol (MSH) produced by Mycothione reductase (MTR). The result showed that thiomeric NPs inhibited MTR by competitive mix model (Figure 5A, 5B) of inhibition and has Ki value of 2.874 and R² value of 0.9988. The enzyme inhibition activity was plotted against the log concentration of thiomeric as shown in Figure 5C. The IC₅₀ of 4.96 was observed for MPTCh-NP.

### 3.5 Nitric oxide generation and cytokine evaluation

Griess assay was performed to measure the nitrite levels after treatment with nanoformulations. Nitrite production was found to be in the order MPTCh-NC > PTCh-NC > TCh-NC > Ch-NC when compared to the control group. MPTCh-NC, PTCh-NC, TCh-NC and Ch-NC treated supernatants of macrophages showed higher levels of nitrite i.e. 80.38 ± 9.89µM, 62.72 ± 7.98µM, 56.15 ± 5.89µM and 50.95 ± 4.79µM respectively (Figure 6A). While Rif and control groups showed 37.93µM and 25.49µM respectively. Coated formulations showed 4.5 and 2.4-fold increase in nitrite production respectively while uncoated formulations showed 1.51-fold higher amount of nitrite when compared to the control group. The increase in nitrite production by MPTCh-NCs treated cells is due to the up regulation of TNF-α which is involved in restricting the mycobacterium inside macrophages [33]. The increased production of TNF-α and IL-12 by coated NCs was in the order MPTCh-NC > PTCh-NC > TCh-NC > Ch-NC when compared to
uncoated Rif and the control group. This clearly demonstrates that coated MPTCh-NC potentiates the spontaneous immunological response by activation of macrophages.

The immunomodulatory activity of the coated nanoformulations was evaluated in terms of TNF-α, IL-10, IL-12 and IL-6 as shown in Figure 6 (B-E) respectively. The coated formulations MPTCh-NC, PTCh-NC, TCh-NC and Ch-NC indicated the TNF-α concentration as 640.84 ± 13.45 pg/ml, 510 ± 10.15 pg/ml, 405.76 ± 8.73 pg/ml and 350.89 ± 7.54 pg/ml respectively and IL-12 concentration of 630.66 ± 13.63, 565.61 ± 15.40, 525.49 ± 10.83 and 390.34 ± 9.59 respectively. The TNF-α levels are significantly higher (p<0.05) as compared to uncoated nanoformulations and control. While IL-6 and IL-10 levels indicated no significant difference (p<0.05) between coated (i.e. MPTCh-NC, PTCh-NC, TCh-NC, Ch-NC), uncoated and control formulations. Stability studies of NC in PBS and FBS was determined at 37°C. No substantial change in nanocarrier size was observed in both PBS and FBS up to 1 week at 37°C as shown in Figure 6F.

3.6 Oxidative spurt and cellular annihilation

Oxidative burst was measured to evaluate the intracellular trafficking of NC towards macrophages after 5 min exposure. ROS was assessed in terms of relative fluorescence units after DCFD staining. According to the data presented in the Figure 6G, the relative fluorescence fervency of PTCh treated cells was much higher than that of control, Ch and TCh after 24 h. It is likely that the oxidative burst may occur at the initial stages of interaction of cells with Mycobacterium. Moreover, the oxidative burst in both the untreated control and M.tb control was very low with no significant differences. ROS is generated as a byproduct of oxygen metabolism. Increased ROS production leads to enhanced bacterial killing via variety of mechanisms such as oxidative defragmentation or affliction of RNA, DNA and bacterial proteins, augmentation of membrane permeability and lipid peroxidation [34]. These mechanisms lead to bacterial death by augmentation of bacterial oxidative phosphorylation [34, 35]. Our findings demonstrated that MPTCh-NCs augmented the generation of ROS.

Different studies are concordant with the fact that there is an involvement of different biochemical, physiological and morphological factors associated with apoptosis in prokaryotes
Cell death initiated after exposure of infected cells with Rif, Ch, TCh, PTCh and MPTCh-NC was detected by annexin-V and PI staining. The increase in the granularity in PTCh and MPTCh-NCs via flow cytometry as shown in Figure 7 (1) A-G depicted the increased uptake of MPTCh-NC and PTCh-NC inside the BCG infected macrophages compared to TCh-NC. The cell annihilation was shown in the Figure 7(2) A-E. Percentage of early and late apoptotic cell death by flow cytometry analysis is given in Table S3. The data suggested the increase in % apoptosis cell treated with different NC compared to control. The increase in apoptosis may be related to DNA stand fragmentation of Mycobacterium infected cells by PTCh and MPTCh. The histograms for the apoptosis data are given in supplementary data Figure S6. The percentage of apoptotic cells increased to 40.4% for PTCh and 33.29% for MPTCh compared to pure drug Rif i.e. 23.25%. This apoptosis is potentially related to the increased ROS production as well as inhibition of MTR.

The resultant fluorescent images demonstrated aberrant margins and dense chromatin with nuclear disintegration shown in supplementary information Figure S7. The treatment of 1µg/ml dose of MPTCh exhibited blue clusters indicating nuclear disintegration after staining with DAPI. These results of DNA disintegration and nuclear protuberances further strengthen the concept of cell demise upon MPTCh exposure.

### 3.7 Drug susceptibility testing and Mycobacterial efflux pump inhibition

The MIC of the Rif, Rif + ver and synthesized NC i.e. Ch, TCh, PTCh and MPTCh-NCs were found to be 2 µg/ml, 0.25 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0.0625 µg/ml and 0.0625 µg/ml respectively for sensitive strain (Table 4). The modulation factor (MF) of 8 and 5.33 showed the synergistic microbicidal potential of verapamil by inhibiting the efflux machinery on bacterial surface. Similarly, MIC of the formulation MPTCh-NCs for resistant strain (R-1343) was found to be 16 µg/ml compared to pure drug (256 µg/ml). The increased MF of PTCh-NCs and MPTCh-NCs with a value of 16 showed the synergistic potential of our NC in Mycobacterial killing. These results confirmed the significantly superior anti-tuberculosis activity by EP inhibition potential of the synthesized nanocargoes.

We also examined the effect of our carrier system at molecular level on gene RV1258 via real time PCR in order to quantify the gene expression level after MPTCh-NC treatment. The results are presented in Figure 8A. There was a substantial 8.13 folds lower expression on RV1258
level after MPTCh-NC treatment in comparison to pure drug Rif. The significant decrease in
gene expression level in comparison to controls and Rif suggests the strong possible evidence of
involvement of our carrier system in *Mycobacterial* efflux pump inhibition. The decreased MIC
values, enhanced modulation factor and decreased expression of RV1258 of as-prepared S-
protected thiomorphic carrier system against resistant strain is potentially due to the inhibition of
the efflux pump (RV1258) which is involved in the expulsion of Rif [7].

### 3.8 *In vivo* bioavailability and stability analysis

Reduced antimycobacterial drug concentrations such as Rif and INH and reduced functional
absorptive area of intestine in TB patients are major concerns limiting the selective and targeted
efficacy of drugs. Therefore, it is significantly important to develop new drug delivery
approaches in order to improve the bioavailability of these drugs. In order to evaluate the oral
bioavailability of prepared MPTCh-NCs, the BALBc mice were orally administered the
marketed Rif and Rif-loaded MPTCh-NCs. Mean plasma drug concentrations against different
time intervals are shown in the Figure 8B. The depicted value of AUC of MPTCh-NCs was
12.31 folds higher in comparison to marketed Rif product. As-prepared MPTCh-NCs
demonstrated effectively increased bioavailability compared to conventionally used products
which make this NC-based platform an ideal therapeutic agent to treat TB while remaining non-
toxic to surrounding tissues/cells.

### 4. Conclusion

This work demonstrated the novel development of S-protected thiomorphic site-specific
nanocargoes (NCs) of Rifampicin (Rif) for the controlled and sustained release of Rif to target
the ROS-mediated cell death. NCs also exhibited sustained and slow release of drug, revealing
the enhanced endosomal escape potential as well as phagolysosomal fusion. Rapid efflux of drug
was demonstrated by the over expression of bacterial efflux proteins (EPs) such as RV1258.
Moreover, *in vitro* studies demonstrated the potential of biocompatible NCs in enhancing
intracellular drug uptake by suppression of macrophage and bacterial efflux machinery as
evident from the increased MF. As-prepared NCs also exhibited improved bioavailability *in vivo*.
These findings provide a proof-of-concept that as-prepared NCs were efficient in eradicating
intracellular pathogens and can be further explored for their immune regulation potential. Taken
together, our findings based on \textit{in vitro} and \textit{in vivo} experiments could be used in solving real-world clinical problems related to TB.

4. Author statement

\textbf{Aisha Rauf}: Conceptualization, investigation, methodology, software, writing and editing; \textbf{Sobia Razzaq}: Software, analysis; \textbf{Tanveer A Tabish}: Reviewing and editing; \textbf{Sabira Tahseen}: Supervision, Resources, Validation; \textbf{Mansoor Abdullah Sandhu}: Resources, validation, investigation, formal analysis; \textbf{Gul Shahnaz}: Supervision, methodology, writing-reviewing and editing.

5. Declaration of competing interest

The authors declare no conflict of interest.
**Table 1:** Characterization of various nanoformulations in terms of mean particle size, polydispersity index (PDI), encapsulation efficiency (EE) and zeta potential

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Diameter (nm)</th>
<th>EE (%)</th>
<th>Zeta potential (mV)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ch-NPs</td>
<td>276 ± 17</td>
<td>73.3 ± 19.2</td>
<td>36.3 ± 2</td>
<td>0.369 ± 0.001</td>
</tr>
<tr>
<td>TCh-NPs</td>
<td>288 ± 13</td>
<td>78.8 ± 12.1</td>
<td>26.2 ± 8.19</td>
<td>0.381 ± 0.4</td>
</tr>
<tr>
<td>PTCh-NPs</td>
<td>381 ± 15</td>
<td>87.6 ± 18.6</td>
<td>24.5 ± 5.13</td>
<td>0.325 ± 0.09</td>
</tr>
<tr>
<td>MPTCh-NPs</td>
<td>390 ± 20</td>
<td>73.68 ± 5.99</td>
<td>18.4 ± 5.46</td>
<td>0.385 ± 0.05</td>
</tr>
</tbody>
</table>

Ch-NPs= Chitosan Rif nanoparticles, TCh-NPs= thiolated chitosan Rif nanoparticles, PTCh-NPs= pre-activated thiolated chitosan Rif nanoparticles, MPTCh-NPs= mannosylated pre-activated thiolated chitosan Rif nanoparticles.

**Table 2:** Drug release data modeling based on in vitro Rifampicin release from various nanoformulations to determine possible drug release mechanisms

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Zero-order</th>
<th>First order</th>
<th>Higuchi model</th>
<th>Korsmeyer-Peppas model</th>
<th>Hixson-Crowell model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C_t=C_0+k_0 t</td>
<td>logQ_0+K_1 t/2.3</td>
<td>f_1=Q=K_H t^1/2</td>
<td>M_i/M_∞=K_t^b+b</td>
<td>3√(W_i+K_{Hc})t</td>
</tr>
<tr>
<td>K_0 R^2</td>
<td>K_0 R^2</td>
<td>K_0 R^2</td>
<td>N R^2</td>
<td>K_0 R^2</td>
<td>N R^2</td>
</tr>
<tr>
<td>pH 7.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ch-NPs</td>
<td>1.92</td>
<td>0.392</td>
<td>0.088</td>
<td><strong>0.984</strong></td>
<td>14.10</td>
</tr>
<tr>
<td>TCh-NPs</td>
<td>1.48</td>
<td>0.533</td>
<td>0.038</td>
<td><strong>0.911</strong></td>
<td>10.69</td>
</tr>
<tr>
<td>PTCh-NPs</td>
<td>1.08</td>
<td>0.296</td>
<td>0.018</td>
<td><strong>0.602</strong></td>
<td>7.445</td>
</tr>
<tr>
<td>MPTCh-NPs</td>
<td>0.69</td>
<td>0.085</td>
<td>0.010</td>
<td>0.123</td>
<td>5.22</td>
</tr>
<tr>
<td>pH 5.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ch-NPs</td>
<td>1.981</td>
<td>0.374</td>
<td>0.097</td>
<td><strong>0.985</strong></td>
<td>14.59</td>
</tr>
<tr>
<td>TCh-NPs</td>
<td>1.68</td>
<td>0.615</td>
<td>0.050</td>
<td>0.970</td>
<td>12.40</td>
</tr>
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<td>PTCh-NPs</td>
<td>1.48</td>
<td>0.727</td>
<td>0.034</td>
<td>0.964</td>
<td>10.43</td>
</tr>
<tr>
<td>MPTCh-NPs</td>
<td>1.26</td>
<td>0.807</td>
<td>0.023</td>
<td>0.942</td>
<td>8.73</td>
</tr>
</tbody>
</table>

Ch-NPs= Chitosan Rif nanoparticles, TCh-NPs= thiolated chitosan Rif nanoparticles, PTCh-NPs= pre-activated thiolated chitosan Rif nanoparticles, MPTCh-NPs= mannosylated pre-activated thiolated chitosan Rif nanoparticles.
Table 3: Comparison of uptake of rifampicin for targeted (mannosylated S-protected thiolated chitosan), S-protected thiolated chitosan, thiolated chitosan and unmodified chitosan and Rif in uninfected and infected macrophages

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Uptake studies (μg Rif/10^6 cells)</th>
<th>Sensitive strain (H37Rv) infected macrophages</th>
<th>Resistant strain (R-1343) infected macrophages</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfected macrophages</td>
<td>Sensitive strain (H37Rv) infected macrophages</td>
<td>Resistant strain (R-1343) infected macrophages</td>
<td></td>
</tr>
<tr>
<td>RIF</td>
<td>3.82 ± 0.05</td>
<td>3.58 ± 0.08</td>
<td>2.146 ± 0.45</td>
<td>0.4974x</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0013 y</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0035z</td>
</tr>
<tr>
<td>Ch-NPs</td>
<td>10.56 ± 0.03</td>
<td>10.87 ± 0.25</td>
<td>5.8 ± 0.01</td>
<td>0.1309x</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0001 y</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0001z</td>
</tr>
<tr>
<td>TCh-NPs</td>
<td>20.67 ± 0.01</td>
<td>20.41 ± 0.21</td>
<td>20.5 ± 0.15</td>
<td>0.1183x</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.1010y</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.99168z</td>
</tr>
<tr>
<td>PTCh-NPs</td>
<td>35.45 ± 0.04</td>
<td>34.89 ± 0.24</td>
<td>35.24 ± 0.01</td>
<td>0.0568x</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.21722y</td>
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<td>0.5708z</td>
</tr>
<tr>
<td>MPTCh-NPs</td>
<td>76.48 ± 0.02</td>
<td>76.88 ± 0.02</td>
<td>76.61 ± 0.05</td>
<td>0.3310x</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>0.5377y</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0812z</td>
</tr>
<tr>
<td>Pretreatment with verapamil</td>
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</tr>
<tr>
<td>RIF</td>
<td>3.85 ± 0.13</td>
<td>3.67 ± 0.17</td>
<td>2.45 ± 0.03</td>
<td>0.119x</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0001 y</td>
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<td>0.0001z</td>
</tr>
<tr>
<td>Ch-NPs</td>
<td>10.98 ± 0.25</td>
<td>10.68 ± 0.34</td>
<td>7.45 ± 0.45</td>
<td>0.134x</td>
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<tr>
<td></td>
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<td>0.000y</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>0.0001z</td>
</tr>
</tbody>
</table>

Both sensitive (H37Rv) and resistant strains (R-1343) of *M. tb* were used to infect the macrophages. The results are expressed as the mean ± SD of three independent experiments. Ch: chitosan, TCh: thiolated chitosan, PTCh: S-protected thiolated chitosan, MPTCh: mannosylated S-protected thiolated chitosan, x: probability value between uninfected macrophages group and sensitive strain infected group, y: probability value between uninfected macrophages group and resistant strain infected group, z: probability value between sensitive strain infected macrophages and resistant strain infected macrophages group.
**Table 4**: Drug susceptibility testing (MIC=µg/ml) of *M. tuberculosis* isolates determined by the BACTEC MGIT 960 system

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rif</th>
<th>Rif+ Ver</th>
<th>Ch</th>
<th>TCh</th>
<th>PTCh</th>
<th>MPTCh</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>2.00</td>
<td>0.25 MF=8</td>
<td>0.5</td>
<td>0.25</td>
<td>0.0625 MF=32</td>
<td>0.0625 MF=32</td>
</tr>
<tr>
<td>R-1343</td>
<td>256</td>
<td>48 MF=5.33</td>
<td>32</td>
<td>32</td>
<td>16 MF=16</td>
<td>16 MF=16</td>
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References


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**Figure 1:** A schematic representation of final structures of mannose coated S-protected thiolated chitosan (MPTCh).

**Figure 2:** (A) Response surface methodology (RSM) plots of the prepared nanocargoes from MPTCh showing the effect of independent factors on (a) size, (b) zeta potential, (c) encapsulation efficiency (EE) and (d) polydispersity index (PDI). (B) Scanning electron microscopy (SEM) analysis of the prepared Ch-NCs, TCh-NCs, PTCh-NCs and rifampicin loaded MPTCh-NCs, at 24,000 X magnification.

Rif=Rifampicin, Ch-NCs=chitosan nanocargoes, TCh-NCs=thiolated chitosan nanocargoes, PTCh-NCs=S-protected thiolated nanocargoes, MPTCh-NCs=mannosylated S-protected thiolated nanocargoes.

**Figure 3:** (A) Proton sponge effect result of Ch, TCh, PTCh and MPTCh polymeric carrier. (B, C) Drug release profile of Rif=Rifampicin, Ch-NCs=chitosan, TCh-NCs=thiolated chitosan, PTCh-NCs=S-protected thiolated chitosan, MPTCh-NCs=mannosylated S-protected thiolated chitosan at (B) Ph=7.4 and (C) pH=5.5. values are mean ± SD of three experiments.

Rif=Rifampicin, Ch-NCs=chitosan nanocargoes, TCh-NCs=thiolated chitosan nanocargoes, PTCh-NCs=S-protected thiolated nanocargoes, MPTCh-NCs=mannosylated S-protected thiolated nanocargoes.

**Figure 4:** Characterization, evaluation and antimycobacterial potential of nanocargoes (A) percentage inhibition of M.tuberculosis at different concentrations of nanocargoes (1-200 µg/ml) (B) IC<sub>50</sub> values of nanocargoes against intracellular macrophages (C) Biocompatibility of Rif and nanocargoes against laboratory isolated mouse peritoneal macrophages after 24 h incubation. All the results are expressed as mean ± SD of the triplicated experiment, and statistically significant differences were evaluated by one-way ANOVA followed by Dunnett’s multiple comparisons test at significance level of **p < 0.05.
Rif=Rifampicin, Ch-NCs=chitosan nanocargoes, TCh-NCs=thiolated chitosan nanocargoes, PTCh-NCs=S-protected thiolated nanocargoes, MPTCh-NCs=mannosylated S-protected thiolated nanocargoes, AO=acridin orange.

**Figure 5:** Mycothione reductase (MTR) inhibition kinetics. (A) Lineweaver-Burk plot of MPTCh, (B) Non linear fitting of mixed model inhibition of MPTCh and (C) Inhibitory activity of Mycothione reductase at different log concentrations of polymer MPTCh and its IC$_{50}$ value.

**Figure 6:** (A) Production of nitric oxide by the *Mycobacterium tuberculosis* infected macrophages after 72h incubation Rif and nanocarriers of Ch-NC, TCh-NC, PTCh-NC and MPTCh-NC, the data. (B, C, D, E) Cytokines evaluation from *Mycobacterium tuberculosis* infected macrophages treated with Rif, Ch-NC, TCh-NC, PTCh-NC and MPTCh-NC by ELISA after 72h incubation. All results are presented as mean ± S.D of experiments performed in triplicate and one-way ANOVA was applied to calculate level of significance. (F) Stability studies of MPTCh-NC in PBS and FBS showing robust stability (Mean ± S.D, n=3) (G) Fluorescent activity via Reactive oxygen species production. (Mean ± S.D, n=3)

ANOVA= Analysis of variance, PBS= Phosphate buffer saline, FBS= Fetal bovine serum, Rif=Rifampicin, Ch-NCs=chitosan nanocargoes, TCh-NCs=thiolated chitosan nanocargoes, PTCh-NCs=S-protected thiolated nanocargoes, MPTCh-NCs=mannosylated S-protected thiolated nanocargoes.

**Figure 7:** (1)Uptake of Nanocargoes by flow cytometry (A) Non- fluorescent mycobacterium (H37Rv) (B) Florescent dsRed BCG strain (C) Uptake of Rifampicin treated dsRed BCG strain (D) Uptake of Ch-NCs treated dsRed BCG strain (E) Uptake of TCh-NCs treated dsRed BCG strain (F) Uptake of PTCh-NCs treated dsRed BCG strain (G) Uptake of MPTCh-NCs treated dsRed BCG strain. (2) Apoptosis study via flow cytometry showing the apoptosis potential of (A) Rif (B) Ch-NCs (C) TCh-NCs (D) PTCh-NCs (E) MPTCh-NCs

Rif=Rifampicin, Ch-NCs=chitosan nanocargoes, TCh-NCs=thiolated chitosan nanocargoes, PTCh-NCs=S-protected thiolated nanocargoes, MPTCh-NCs=mannosylated S-protected thiolated nanocargoes.
Figure 8: (A) Gene expression level of RV1258 by real time PCR. All results are presented as mean ± S.D of experiments performed in triplicate and one-way ANOVA was applied to calculate level of significance. (Mean ± S.D, n=3) (B) Plasma concentration V/S time curve plot of commercial Rif and MPTCh-NCs. All results are presented as mean ± S.D of experiments performed in triplicate (Mean ± S.D, n=3)

MPTCh-NCs=mannosylated S-protected thiolated nanocargoes, D271= Rif resistant mycobacterial strain, H37Rv= wild type mycobacterial strain, Rif= Rifampicin
Figure 2
Figure 3
Figure 4
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Figure 8
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: