Pharm Res
DOI 10.1007/s11095-017-2206-3

RESEARCH PAPER

Conjugated and Entrapped HPMA-PLA Nano-Polymeric Micelles Based Dual Delivery of First Line Anti TB Drugs: Improved and Safe Drug Delivery against Sensitive and Resistant *Mycobacterium Tuberculosis*

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Received: 29 April 2017 / Accepted: 8 June 2017

ABSTRACT

Purpose First line antiTB drugs have several physical and toxic manifestations which limit their applications. RIF is a hydrophobic drug and has low water solubility and INH is hepatotoxic. The main objective of the study was to synthesize, characterize HPMA-PLA co-polymeric micelles for the effective dual delivery of INH and RIF.

Methods HPMA-PLA co-polymer and HPMA-PLA-INH (HPI) conjugates were synthesized and characterized by FT-IR and ^{1}H–NMR spectroscopy. Later on RIF loaded HPMA-PLA-INH co-polymeric micelles (PMRI) were formulated and characterized for size, zeta potential and surface morphology (SEM, TEM) as well as critical micellar concentration. The safety was assessed through RBC's interaction study. The prepared PMRI were evaluated through MABA assay against sensitive and resistant strains of *M. Tuberculosis*.

Results Size, zeta and entrapment efficiency for RIF loaded HPMA-PLA-INH polymeric micelles (PMRI) was 87.64 ± 1.98 nm, −19 ± 1.93 mV and 97.2 ± 1.56%, respectively. *In vitro* release followed controlled and sustained delivery pattern. Sustained release was also supported by release kinetics. Haemolytic toxicity of HPI and PMRI was 8.57 and 7.05% ($p < 0.01$, INH *Vs* PMRI; $p < 0.0001$, RIF *Vs* PMRI), respectively. MABA assay (cytotoxicity) based MIC values of PMRI formulation was observed as ≥0.0625 and ≥0.50 μg/mL (for sensitive and resistant strain). The microscopic analysis further confirmed that the delivery approach was effective than pure drugs.

Conclusions RIF loaded and INH conjugated HPMA-PLA polymeric micelles (PMRI) were more effective against sensitive and resistant *M tuberculosis*. The developed approach can lead to improved patient compliance and reduced dosing in future, offering improved treatment of tuberculosis.

KEY WORDS co-polymeric micelles · HPMA-PLA conjugate · MABA · tuberculosis

ABBREVIATIONS

Ar	Aromatic
CMC	Critical micelle concentration
DCC	N,N′-dicyclohexylcarbodiimide
DCM	Dichloromethane
EDC	N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide
ETH	Ethambutol
HPI	HPMA-PLA-INH conjugates
HPMA	N(2-hydroxypropyl) methacrylamide
INH	Isoniazid
MDR-TB	Multidrug-resistant TB
PLA	Poly lactic acid
PMRI	RIF loading in HPMA-PLA-INH conjugates
PYZ	Pyrazinamide
RIF	Rifampicin
SEM	Scanning electron microscopy and
TB	Tuberculosis
TEM	Transmission electron microscopy

Electronic supplementary material The online version of this article (doi:10.1007/s11095-017-2206-3) contains supplementary material, which is available to authorized users.

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Published online: 06 July 2017

INTRODUCTION

Tuberculosis (TB) is a lethal infectious respiratory disorder. Almost 30% of the world population is affected with TB. According to WHO reports, after the human immune deficiency virus, TB is considered as the second leading cause of death. TB is caused by different strains of gram negative (−ve) bacteria i.e. Mycobacteria, in which *Mycobacterium tuberculosis* being the most common strain which may affect any part of the human body. More than 80% of infections are the cases of pulmonary TB. Extra-pulmonary TB has also been reported as a result of inhaled *M. tuberculosis* in the form of bacilli, most of which exists in Asia and Africa compared to Europe and American countries. Patients with supressed immunity are more prone to tuberculosis infection. The main cause of tuberculosis is the (due to long course of therapy) poor patient compliance and failure of uptake of the drug regimen due to systemic toxicity (1). Worldwide, 9.6 million people are estimated to have fallen ill with TB in 2014, in which 5.4 million were men, 3.2 million were women and 1.0 million were children (2). The first line treatment includes drugs such as Rifampicin (RIF), Isoniazid (INH), Pyrazinamide (PYZ) and Ethambutol (ETH). The 6-month regimen includes INH, RIF, PYZ and ETH given daily or intermittently for 2 month, followed by RIF and INH for 4 months' treatment-limiting side effects fluctuate with minimum severity. Mainly, INH and RIF are given for the effective management of TB. INH is a pyridine-4-carbohydrazide, a hydrazide derivative of isonicotinic acid. Officially, INH is official drug in various monographs such as, Indian Pharmacopoeia (IP), British Pharmacopoeia (BP), United States Pharmacopoeia (USP), Japanese Pharmacopoeia (JP) and European Pharmacopoeia (EP). INH acts as bactericidal for the actively multiplying microbe and bacteriostatic for "resting" bacilli. INH is metabolised by mycobacterial catalase-peroxidase into an active metabolite, so INH acts as a prodrug. INH exhibits its activity by inhibiting the bio-synthesis of mycolic acid (3). RIF is a first line antibacterial drug. It is also a derivative of rifamycin B, acts as an inhibitor of DNA dependent RNA polymerase of bacteria by binding strongly to their beta subunits (4).

Long term administration of anti TB drugs leads to poor patient compliance, which is the most common reason for chemotherapy failure in TB. So, combined delivery of first line anti TB drugs can help to minimise toxicity as well as to improve patient compliance. The combined delivery is also a good strategy, as well as it is aimed to be minimise the dosing frequency of anti TB drugs through the administration of multidrug therapy for better patient compliance (5). The emergence of multidrug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) have been seen from last two decades. There is an urgent need for drugs that can shorten the duration of TB chemotherapy and are active against drug-resistant strains of *M. tuberculosis*. It is imperative to discover and develop potent small molecule inhibitors of *M. tuberculosis* that have a novel mode of action and are active against both drug-sensitive and drug-resistant TB (6). All the above problems can be solved with nano-carrier based delivery such as using nanoparticles, dendrimers, liposomes and polymeric micelles etc. Most of the nano-carriers are nanosized synthetic molecules used to cross the biological barrier. Nano-carriers are used to deliver the pharmaceutical drugs because of their promising features over conventional carriers i.e. (i) have ability to encapsulating the drugs without any effect on it, (ii) release of drugs at target site under appropriate condition, (iii) to deliver the drugs actively or passively to a target site (7). As the polymeric micelles are used as a novel drug carrier for poorly water soluble drugs and serve as targetable drug carriers. Polymeric micelles have attracted significant attention in the field of drug and gene delivery due to their excellent biocompatibility, low toxicity, enhanced blood circulation time and ability to solubilize a large number of drugs in their micellar core (8). Polymeric micelles have nanometric size (size ranges from 10 to 200 nm) and long circulation time. Polymeric micelles are an important and attractive class of drug carriers, useful for the *IV* administration of hydrophobic drugs (9).

Micelles were synthesized from two polymers i.e. biocompatible and biodegradable block co-polymers and show functional groups on their surfaces for attachment (10). The main features of co-polymeric micelles are self-assembling of amphiphilic block co-polymer molecules and encapsulation by means of physical mixing instead of chemical conjugation (11). Hydrophobic drug molecules can be incorporated in the inner core of polymeric micelles and micelles inhibit the inter-miceller aggregation of hydrophobic cores with a hydrophilic outer shell layer which works as a barrier against inter-miceller aggregation, so they maintain water solubility. The advantage of polymeric micelles is that various chemical species can easily binds with the micelles (12).

In the present work, we attempted to formulate HPMA-PLA co-polymeric micelles of isoniazid and rifampicin (Fig. 1a; schematic representation) for dual delivery at the same time against different strains of *M. tuberculosis*. The manuscript also reported the related evidences of better effectivity of the prepared formulation over the naïve drugs.

MATERIAL AND METHODS

Materials

INH and RIF were obtained as gift samples from Kwality Pharmaceutical Limited, Amritsar, Punjab, India. PLA (poly lactic acid) was also a gift sample obtained from Evonik Industries, Germany. Dichloromethane (DCM) and sodium

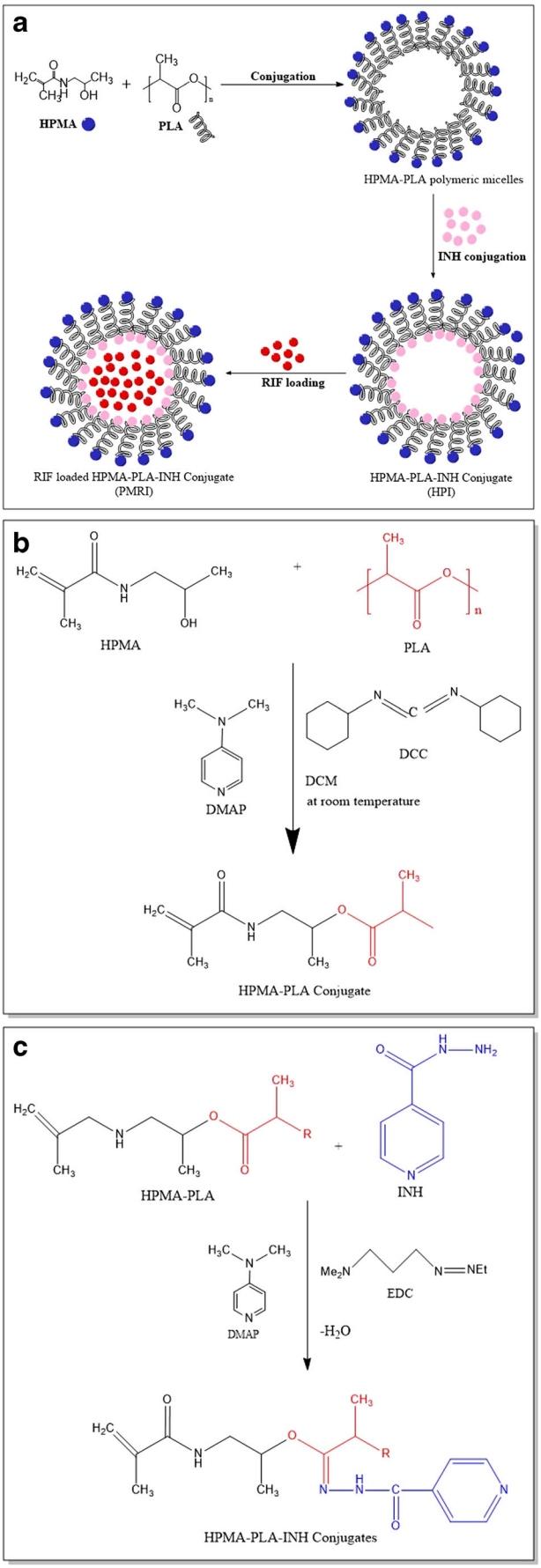


Fig. 1 (**a**) Schematic representation of formation of RIF loaded HPMA-PLA-INH (PMRI) co-polymeric micelles. [INH: Isoniazid, RIF: Rifampicin]. (**b**) Synthetic scheme of HPMA-PLA co-polymer. (**c**) Synthetic scheme of conjugation of INH with HPMA-PLA co-polymer.

hydrogen carbonate were purchased from CDH chemicals, India. N,N′-dicyclohexylcarbodiimide (DCC), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) anhydrous and sodium sulfate were procured from Hi-Media, India. DMAP (4-dimethylaminopyriine), dialysis membrane (5 kDa, MWCO) were procured from Hi-media Laboratories, Mumbai, India. 1-aminopropan-2-ol and methacryloyl chloride were procured from Alfa Aesar. The inoculum was provided by the Japanese Leprosy Mission for Asia (JALMA), Agra and 96 well plates were purchased from Tarsons, India. All the other chemicals, solvents and reagent were of analytical grade and were used as such without further purification.

Synthesis and Characterization of [N (2-Hydroxypropyl) Methacrylamide] (HPMA)

HPMA was synthesized following previously reported method (13). Briefly, anhydrous sodium hydrogen carbonate (1 g, 11.9 mmol) and 1-aminopropan-2-ol (0.89 g, 11.9 mmol) solution was dissolved in dichloromethane (DCM) and cooled at 0°C. Methacryloyl chloride (1.2 g, 11.9 mmol) in DCM was added drop-wise under cooling and vigorous stirring for 1 h. The reaction mixture was again stirred for 30 min. at 15°C, then anhydrous sodium sulfate (1.6 g, mmol) was added, the mixture was filtered and dried. Under reduced pressure, the dry filtrate was concentrated approximately to half of its volume. By crystallization at −20°C, HPMA was procured from DCM and from acetone the pure HPMA was obtained by recrystallization. The characterization of HPMA was done by FT-IR and ^{1}H–NMR spectroscopy. FT-IR analysis was done by using KBr pellet method (Perkin Elmer spectrophotometer, M/s Perkin Elmer Co., Waltham, Massachusetts, USA). Using $CDCl_3$ as a solvent, proton ^{1}H–NMR spectroscopy was performed in Bruker Ascend 500 MHz NMR spectrometer, Switzerland. ^{1}H–NMR spectrum was recorded considering 7.269 ppm for ^{1}H–NMR as internal standard.

Synthesis and Characterization of HPMA-PLA Conjugates

Poly-L-lactic Acid (PLA) (500 mg, 0.05 mmol) was dissolved in DCM (5 mL) with HPMA (7.1 mg, 0.05 mmol) in the excess of DCC (10.31 mg, 0.05 mmol). The mixture was stirred for 24 h in the presence of DMAP (6.1 mg, 0.05 mmol) converted into HPMA-PLA conjugates **(**Fig. 1b**)**. The conjugation was dialysed in 5 kDa membrane for 24 h to remove the unconjugated reactants (14). The characterization of HPMA-PLA

co-polymer was done by FT-IR using KBr pellet method (Perk, M/s Perkin Elmer Co., Waltham, Massachusetts, USA). ^{1}H–NMR spectroscopy was performed in Bruker Ascend 500 MHz NMR spectrometer, Switzerland using $CDCl_3$ as a solvent. ^{1}H–NMR spectrum was recorded considering 7.269 ppm for ^{1}H–NMR as internal standard.

Synthesis and Characterization of HPMA-PLA-INH (HPI) Conjugates

HPMA-PLA (100 mg, 0.009 mmol) was dissolved in DCM (5 mL) in the presence of DMAP (1.099 mg, 0.009 mmol) followed by the addition of INH (1.23 mg, 0.009 mmol) dropwise under nitrogen atmosphere at room temperature, then EDC (1.39 mg, 0.009 mmol) was added and mixture was stirred for 24 h. The conjugated INH with co-polymer was synthesized (Fig. 1c**)** and characterized (15). The conjugation of INH with HPMA-PLA co-polymer was characterized by FT-IR using KBr pellet method (Perk, M/s Perkin Elmer Co., Waltham, Massachusetts, USA) and ^{1}H–NMR spectroscopy using $CDCl_3$ as a solvent, proton NMR spectroscopy was performed in Bruker Ascend 500 MHz NMR spectrometer, Switzerland. ^{1}H–NMR spectrum was recorded considering 7.269 ppm for ^{1}H–NMR as internal standard.

Critical Micellar Concentration (CMC)

To determine the CMC, iodine was used as a hydrophobic probe. Briefly, standard solution of KI/I_2 was prepared by dissolving iodine (250 mg) and potassium iodide (500 mg) in deionized water (25 mL). A set of dilutions of HPMA-PLA co-polymer in deionized water were prepared. In each dilution, 25 μL of KI/I_2 standard solution was added, the mixture was kept into the dark for 12 h at room temperature, than absorbance of dilutions was recorded in UV-Visible spectrophotometer (Labtronics, India) at 366 nm. The graph was plotted between absorption intensity and polymer-mass concentration. The concentration of the polymer where there is sharp increase in absorbance was detected and considered as CMC value (16).

Rifampicin Loading in HPMA-PLA-INH Conjugates (PMRI)

Rifampicin loaded HPMA-PLA-INH (PMRI) micelles were prepared by direct dissolution method. Briefly, the formation is induced by adding two solutions to suitable drug-polymer ratio. This process involves dissolving the HPI conjugates along with the drug in distilled water (10 mL). RIF (10 mg) dissolved in acetonitrile and was added dropwise in aqueous solvent, the mixture of solutions was stirred for 24 h in round bottom flask at room temperature (17). Characterization of PMRI was done by size analysis, polydispersity index, zeta potential and surface morphology (SEM, TEM)

CHARACTERIZATION

Size, Zeta Potential and Polydispersity Index

Photon correlation spectroscopy (Nano ZS, Malvern, UK) was used to determine the mean particle size, zeta potential and polydispersity index of PMRI. Briefly, the samples were diluted appropriately using deionized water, measurement of the samples was performed in triplicate with fixed angle of 90° at 25°C temperature with the help of electrophoretic cell (18).

Surface Morphology & Electron Microscopy

Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) techniques were used to determine the shape, size and morphology of the prepared PMRI micelles. SEM analysis was performed at Malaviya National Institute of Technology (MNIT), Jaipur, Rajasthan, India (Nova Nano SEM 450, FEI) (19). TEM analysis was performed using copper grids stained with phosphotungstic acid solution (2%). A drop of diluted sample was placed on this copper grids for 30 s and dried at room temperature and observed in electron microscope (FEI, Japan, Model techani G^2) at Sophisticated Advanced Instrument Facility, All India Institute of Medical Science (SAIF, AIIMS), New Delhi, India (20).

Entrapment Efficiency and Drug Loading

Drug entrapment efficiency and drug loading were determined by UV-Vis spectroscopy. Weighed amount of PMRI was dissolved in phosphate buffer (PBS pH 7.4) and kept overnight for continuous shaking at room temperature followed by centrifugation. After centrifugation, supernatant liquid was absorbed at 262 and 334 nm by UV-Vis spectroscopy. Drug entrapment efficiency and drug loading efficiency were expressed as the percentage of drug amount in co-polymeric micelles to the initial feed drug quantity (21,22).

$$\%\textit{Entrapment efficiency} = \left[\textit{Mass of drug in micelles} \Big/ \textit{Mass of feed drug}\right] \times 100$$

$$\%\textit{Loading efficiency} = \left[\textit{Weight of drug in micelles} \Big/ \textit{Weight of micelles}\right] \times 100$$

In Vitro Drug Release and Mathematical Kinetic Modelling

The in-vitro release of INH and RIF from PMRI micelles was performed in phosphate buffer at pH 7.4 using membrane dialysis method. Briefly, 2 mL of drug loaded micelles were suspended in dialysis membrane (MWCO; 50 kDa, Hi-media) and kept in 100 mL of buffer at room temperature with continuous stirring at 100–150 rpm (Remi, India). After a predetermined time interval, 2 mL of PBS solution was withdrawn and replaced with the equal volume of fresh PBS buffer. Withdrawn samples were scanned at 262 and 334 nm, the amount of released INH and RIF was determined based on the absorbance. The release of drugs (INH and RIF) was compared using UV-Vis spectroscopy graphs (15,19).

Five different kinetic models like zero order, first order, Higuchi, Hixon Crow-well and Korsmeyer-Peppas model were used to study the release kinetic model. The obtained in-vitro drug release data was fitted in five different kinetic models.

Ex-Vivo Hemolytic Toxicity

Hemolytic toxicity of prepared PMRI micelles and drugs were executed. Briefly, the whole blood was collected from a healthy volunteer (5 mL) and stored in EDTA anticoagulant storage vial. Then, RBCs were separated using centrifugation at approximately. 1350 rpm (R-4C DX, REMI, India) and suspended in normal saline (0.9% *w/v*). In normal saline, different-different samples (20 ppm) were prepared, each sample in 1 mL quantity was added to the RBC's. The mixture was stirred continuously for 1 h and samples were centrifuged and supernatant was taken and diluted with saline. Absorbance was observed at wavelength of 540 nm. As a control, distilled water was used to RBC's suspension for which hemolytic value was considered as 100%.

$$\%Hemolysis = \left[Ab_s \Big/ AB_{100}\right] \times 100$$

Where Ab_s is the absorbance of the sample and AB_{100} is the absorbance of control without formulation (23). Microscopic study of hemolysed RBCs was performed using fluorescence microscope at 40x (Olympus Inverted Fluorescence Microscope CKX53, Japan).

Cytotoxicity and Microscopy

Cytotoxicity studies of prepared PMRI micelles were performed to check the anti-TB potential of prepared nano formulations. The cytotoxicity of drug loaded formulation was evaluated through MABA (Microplate-Based Alamar Blue Assay). The study was performed at National Japanese Leprosy Mission for Asia (JALMA) Institute of Leprosy and Other Mycobacterial Diseases, Agra, India.

In this study, to minimize evaporation of the medium, sterile deionized water (200 µL) was added all outer-perimeter wells of sterile 96-well plates during incubation. 7H9GC broth (100 mL) was added in rows B to G in columns 3 to 11 of wells. Drug solutions (100 µL) were added to the wells in rows B to G in columns 2 and 3. From column 3 to column 4, by using a multichannel pipette, 100 mL was transferred and mixed properly. Through column 10, serial dilutions (1:2) were continued and excess medium from column 10 was withdrawn. By using an eppendorf repeating pipette, *M. tuberculosis* inoculum (100 µL) was added in B to G rows of columns 2 to 11. Now, column 11 was worked as drug-free (inoculum-only) controls. With the help of parafilm, the plates were sealed and incubated for 5 days at 37°C. Freshly prepared mixture of Alamar Blue (Accumed International, Westlake, Ohio) reagent and 10% Tween 80 (50 µL) was added to B11. Reincubation of plates was done for more 24 h at 37°C, then the colour change of wells was observed, if the well showed pink colour from blue, in microplate, all the wells were filled with reagent mixture and again incubated for 24 h at room temperature. After incubation, the colour change of wells was evaluated. Pink colour indicated the the growth while the blue colour showed no growth of the respective MTBs. If a well showed violet colour, it turned pink after another day of incubation. The lowest drug concentration which prevented a colour change from blue to pink, was considered as MIC and the obtained results were further confirmed through microscopic examination (24).

Statistical Data Analysis

All the experiments were performed at least three times. Student's t test was used for statistical treatment of data using GraphPad Prism (version 7.0, CA, USA) software, with $p < 0.05$ considered to be significant differentiate.

RESULTS AND DISCUSSION

Synthesis and Characterization of HPMA

HPMA was synthesized and characterized by FT-IR and proton (^{1}H) NMR spectroscopy. The obtained spectra confirmed the formation of HPMA. In FT-IR, a peak of amide bond detected at 1653.04 cm^{-1}. Another peak was observed at 3299.74 cm^{-1} for sp^3 carbon (alkyl/methyl group), and at 2974.29 cm^{-1} for sp^3 carbon (alkenes). HPMA showed the characteristic shift for proton of amide at 7.7 ppm (s,1H, −CO-NH-), 5.3 ppm of alcohol (s, 1H, −OH) and confirmed the presence of methyl at 1.2 ppm (t, 3H, $-C-CH_3$) protons, respectively (Supporting data Fig. S1, Fig. S2).

Synthesis and Characterization of HPMA-PLA Conjugates

HPMA-PLA co-polymer was synthesized and characterized by FT-IR and proton ^{1}H–NMR spectroscopy. FT-IR spectroscopy conforms the conjugation, 1747 cm^{-1}, showed the presence of ester bond and at 3383 cm^{-1} that confirmed the presence of amide (N-H). Amide (C = O) and alkene (C = C) peaks were observed at 1660 and 1624 cm^{-1}, respectively, **(**Fig. 2a**)**. In FT-IR spectra, all the observed peaks confirmed the conjugation of HPMA-PLA. Like FT-IR spectroscopy, the conjugation of HPMA-PLA co-polymer was further confirmed through proton nuclear magnetic resonance (^{1}H–NMR) spectroscopy. The conjugation of HPMA-PLA signified the characteristic shift at 8.262 ppm (s, 1H, −CO-NH-) which reveals that proton in amide group on structural moiety. Chemical shift at 4.194 ppm (m, 1H, −CH) indicates the presence of methine group which conjugated with α -O-C = O (ester group) that conforms formation of ester bond **(**Fig. 2b**)**. Chemical shift of amide proton of HPMA group was deshielded might be coupling with PLA and formed an ester bond in HPMA-PLA conjugation.

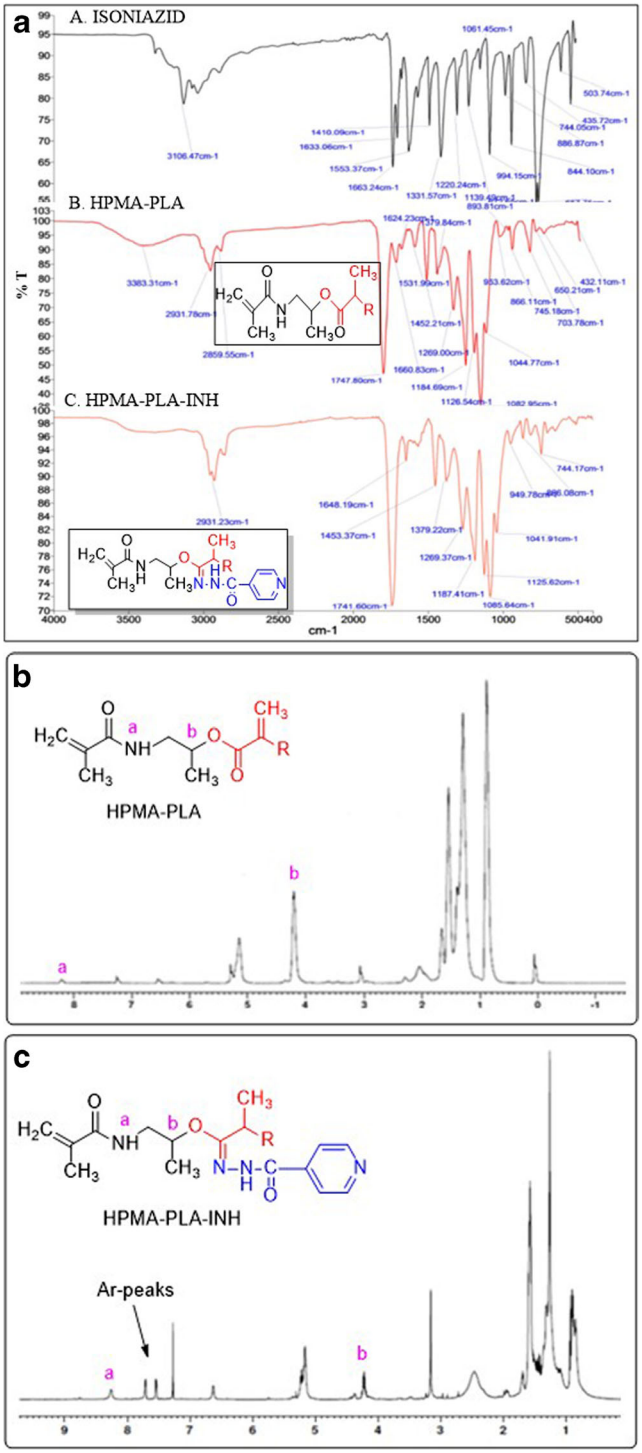


Fig. 2 (**a**) FT-IR spectra of; *A* naïve INH, *B* HPMA-PLA conjugates, *C* HPMA-PLA-INH (HPI) conjugates. (**b**) ^{1}H–NMR spectrum of HPMA-PLA conjugate. (**c**) ^{1}H–NMR spectrum of HPMA-PLA-INH (HPI) conjugate.

Synthesis and Characterization of HPMA-PLA-INH (HPI) Conjugates

The conjugation of INH with HPMA-PLA co-polymer was synthesized and characterized by FT-IR and proton ^{1}H–NMR spectroscopy. Major peak of conjugated formulation observed at 1648 cm^{-1} i.e. a weak peak, refers to C = N (imines) and at 2931 cm^{-1} that confirmed the presence of C-H (alkanes) **(**Fig. 2a**)**. Further confirmation of conjugation of INH with co-polymer was done through proton nuclear magnetic resonance (^{1}H–NMR) spectroscopy. The ^{1}H–NMR proton spectrum of HPI conjugation showed chemical shift at 8.262 ppm (s, 1H, −CO-NH-) for amide proton of HMPA-PLA conjugate and chemical shift at 4.194 ppm (m, 1H, -CH-) exhibiting the CH proton of HPMA and moreover, characteristics chemical shift at 7.526 (d, 2H, −Ar CH) (J = 5.5)Hz and 7.714 (d, 2H, −Ar CH) (J = 5.5)Hz for the -CH group of 4-pyridine moiety (an aromatic ring) revealed confirmation for the conjugation of INH to the HMPA-PLA co-polymer **(**Fig. 2c**)**. INH also contains an amide group, hence ^{1}H–NMR spectrum displayed two chemical shifts for amidic proton, while in HMPA-PLA conjugation, it shows chemical shift only for -NHCO- group.

Critical Micellar Concentration (CMC)

CMC of the co-polymeric micelles was determined by iodine method in which iodine was used as a hydrophobic probe. CMC of the co-polymeric micelle affect its *in vivo* and *in vitro* stability. Conversion of I_3 into I_2 (in the excess of KI) felicitated I_2 solubilization in the hydrophobic microenvironment of the synthesized HPMA-PLA co-polymer. It maintained the concentration of I_2 and the graph of absorption intensity of I_2 was plotted between concentration (μg/mL) and absorbance in Fig. 3 and the CMC value was obtained 35 μg/mL and at this concentration, the micelles were formed (16,25). The result of critical micellar concentration of HPMA-PLA co-polymeric micelles was reported for the first time.

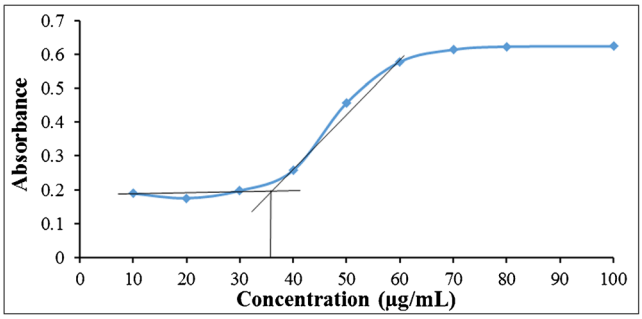


Fig. 3 CMC plot as a function of copolymer concentration (HPMA-PLA) in µg/mL.

Size, Polydispersity Index and Zeta Potential

The mean size and zeta potential of drug loaded co-polymeric micelles (PMRI) were 87.64 ± 1.98 nm and −19 ± 1.93 mV and mean size and zeta potential of HPMA-PLA-INH conjugates (HPI) was 111.2 ± 2.34 nm and −45.3 ± 2.30 mV, respectively. The size of RIF loaded co-polymeric micelles can be observed in Table 1. The PMRI and HPI conjugates showed a larger size.The findings indicated the conjugation/encapsulation of both the bio-actives within the micelles. Polydispersity index of PMRI and HPI were obtained 0.374 ± 0.17 and 0.546 ± 0.10 which represents that how broad the distribution is around the mean particle size (26).

Surface Morphology & Electron Microscopy

The morphology of PMRI was observed by SEM and TEM. The micelles were formed to have uniform surface morphology with spherical shape. SEM images confirmed about the homogenous nature of particles with a uniform distribution (19) **(**Fig. 4a**)**. TEM micrographs further confirmed the size and distribution as well as morphology of micelles **(**Fig. 4b**)**. The results were supported by the earlier studies, it explained that the size of RIF loaded co-polymeric micelles was less than RIF free co-polymeric micelles (27). The average particle size of HPI and PMRI were observed around 100 nm.

Table 1 Comparative data for the particle size, zeta potential, polydispersity index, and entrapment efficiency of optimized formulations [HPI: HPMA-PLA-INH conjugates, PMRI: Rifampicin loaded HPMA-PLA-INH co-polymeric micelles]

Formulation Code	Average Particle Size (nm)	Zeta Potential (mV)	Polydispersity Index (pdi)	Entrapment Efficiency (%)
HPI	111.2 ± 2.34	−45.3 ± 2.30	0.546 ± 0.10	–
PMRI micelles	87.64 ± 1.98	−19.0 ± 1.93	0.374 ± 0.17	97.2 ± 1.56

Entrapment Efficiency and Drug Loading

RIF entrapment efficiency of PMRI was 97.2 ± 3.56% while loading was 32.4 ± 1.56% and INH conjugation efficiency was observed 71.54 ± 1.23%. These results are supported by the earlier reported studies of polymeric micelles (26), as the RIF is a hydrophobic drug and it is good to have higher content of PLA in co-polymer for entrapment of RIF in micelles. RIF can be easily loaded in the hydrophobic core due to hydrophobic interactions. The compatibility of polymeric micelles hydrophobic core and hydrophobic drug RIF was depended on PLA side chain.

In Vitro Drug Release and Kinetic Modelling

In vitro release study of prepared formulations (HPI and PMRI) and pure drugs (INH and RIF) was performed by membrane dialysis method. The results of the study revealed that, pure INH and RIF were released in a burst manner in approximately 2 h and after that concentration was constant while the release of conjugated INH and loaded RIF from micelles was observed more than 36 h and after that concentration was constant **(**Fig. 5a**)**. Loaded RIF released faster from micelles in comparison to the conjugated INH in HPMA-PLA-INH. INH have hydrazine group which was covalently conjugated with PLA. INH release will lead to bond breakage (15), while loaded RIF was released as burst from co-polymeric micelles. The comparative study is shown in Fig. 5b. Result of release of RIF was supported by the previous reported results (19) that explained release rate of RIF was fast in the initial stage and the rate of release of RIF slowed down at later time in the encapsulated form. The rate of release was also reduced due to increased PLA contents.

The *in vitro* release of drug from PMRI micelles was easily explained by Korsmeyer-Peppas model **(**Fig. 5c**)**, the mathematical linear kinetic modelling presented highest linearity [r^2 = 0.8197, INH) & 0.8803, RIF], described in Table S1 Korsmeyer-Peppas model described the magnitude of release exponent "N" which explained that the release mechanism was case-II relaxational release having stresses in hydrophilic polymers which swell in water or biological fluids (28).

Ex-Vivo Hemolytic Toxicity

Hemolytic toxicity of the prepared co-polymeric micelles, conjugates and pure drugs was performed. The results obtained showed that the toxicity of PMRI was reduced in comparison with free drugs (INH, RIF). The hemolytic toxicity of RIF and INH were observed to be 19.6% and 14.1% ($p < 0.005$, INH *vs* HPI), respectively, while the toxicity of HPI and PMRI were observed 8.57% and 7.05% ($p < 0.01$, INH *vs* PMRI; $p < 0.0001$, RIF *vs* PMRI), respectively, which is less than half the toxicity of pure drugs **(**Fig. 6a**)**. To reduce the toxicity of

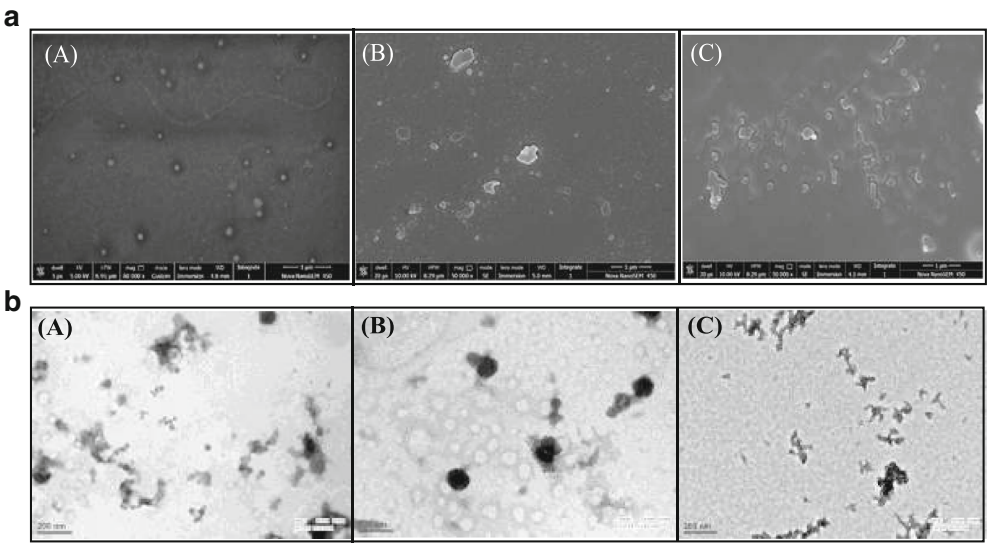


Fig. 4 **(a)** Scanning electron (SEM) photomicrograph of; *A* HPMA-PLA conjugates, *B* HPMA-PLA-INH (HPI) conjugates, *C* rifampicin loaded HPMA-PLA-INH (PMRI) co-polymeric micelles (The scale bar represents 1 μm). **(b)** Transmission electron (TEM) photomicrograph of; *A* HPMA-PLA conjugates, *B* HPMA-PLA-INH (HPI) conjugates, *C* Rifampicin loaded HPMA-PLA-INH (PMRI) co-polymeric micelles (scale bar =200 nm).

drug, HPMA acted as a shell forming segment because of its physicochemical properties including high water solubility, low toxicity as well as having multiple functional groups. As per previous results of nanoparticulate formulations of antineoplastic drugs the hemolytic toxicity can be reduced because of reduced direct contact of the drugs with RBCs. The results revealed that RIF is more hemolytic than the INH and block copolymer as well, but in the micellar form RIF was masked within the hydrophobic environment of micelles leading to the reduced hemolytic toxicity (18,29,30).

Microscopic Analysis RBC Hemolysis

The results obtained were further confirmed through microscopic examination at 10 and 40X magnification

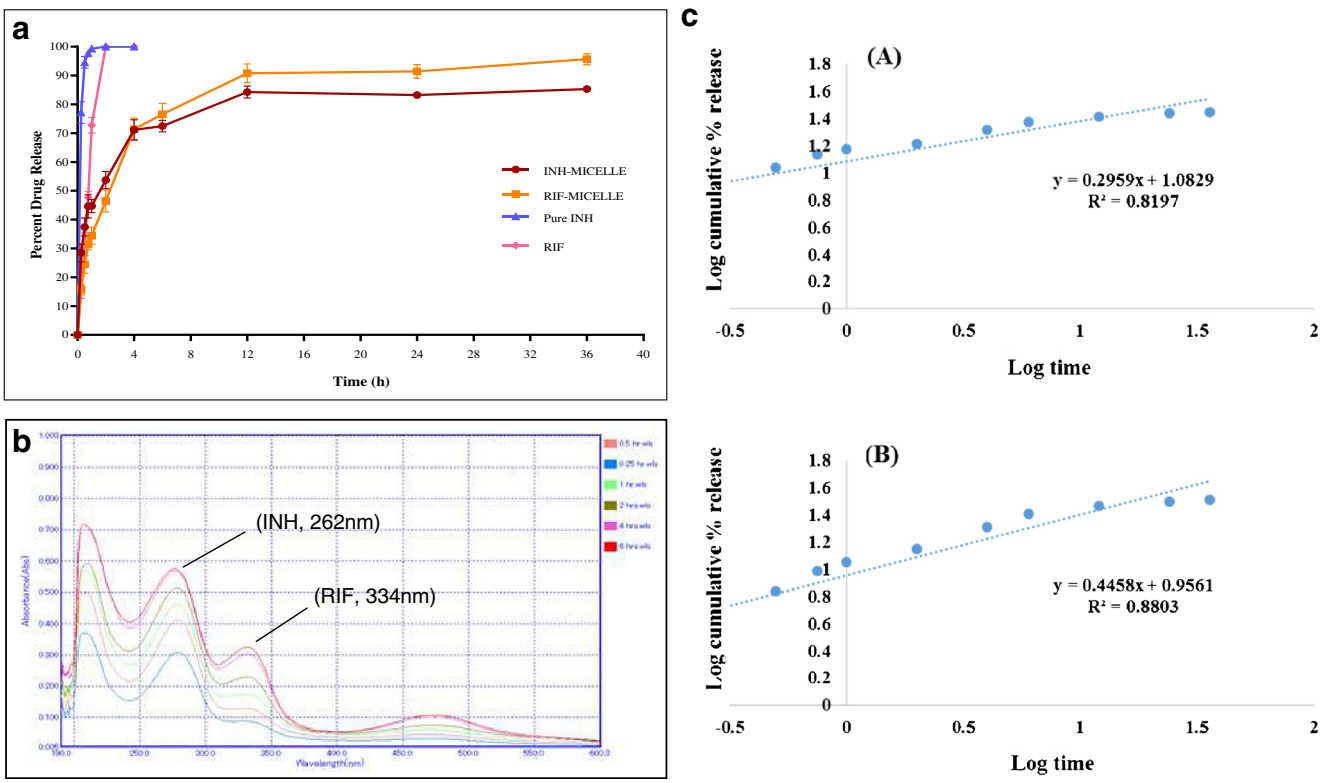


Fig. 5 **(a)** *In vitro* drug release from drug loaded co-polymeric micelles (HPI & pure drug) at PBS buffer pH 7.4 (n = 3). [INH: Isoniazid, RIF: Rifampicin, HPI: HPMA-PLA-INH conjugates, PMRI: Rifampicin loaded HPMA-PLA-INH co-polymeric micelles]. **(b)** Comparative *in vitro* of INH and RIF in PBS buffer at pH 7.4 in UV-Vis spectroscopy. **(c)** Graphical representation of Korsmeyer-Peppas kinetic release model for;(*A*) INH and (*B*) RIF.

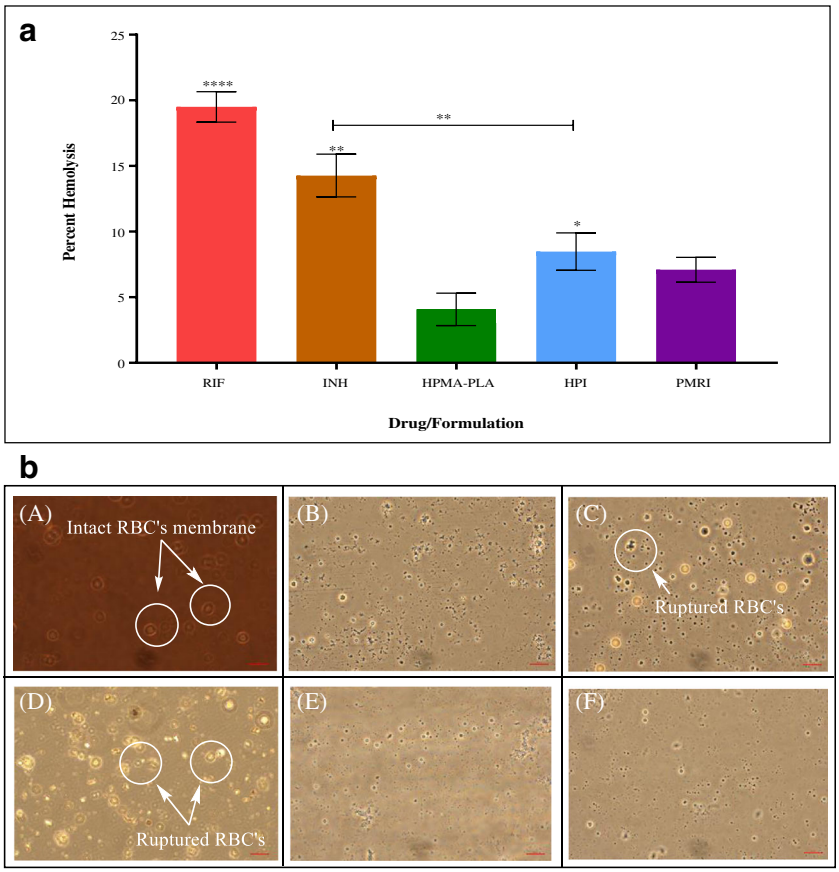


Fig. 6 (**a**) Percent hemolysis of various formulations. Values represents mean ± SD ($n = 3$). [INH: Pure Isoniazid, RIF: Pure Rifampicin, HPI: HPMA-PLA-INH conjugates, PMRI: Rifampicin loaded HPMA-PLA-INH co-polymeric micelles]. * indicates less significant difference with p value = 0.0122 (HPI *Vs* PMRI). ** indicates significant difference with p value = 0.0014 (INH *Vs* HPI). ** indicates significant difference with p value = 0.0098 (INH *Vs* PMRI). **** indicates extremely significant difference with p value = <0.0001 (RIF *Vs* PMRI). (**b**) Microscopic images of drugs as well as formulation effected RBC's at 40X. *A* Pure RBC's *B* Distilled water treated RBC's *C* INH treated RBC's *D* RIF treated RBC's *E* HPI treated RBC's & *F* PMRI treated RBC's. [INH: Pure Isoniazid, RIF: Pure Rifampicin, HPI: HPMA-PLA-INH conjugates, HPI: HPMA-PLA-INH conjugates, PMRI: Rifampicin loaded HPMA-PLA-INH co-polymeric micelles].

under microscope. The microscopic study of samples (prepared for hemolytic study) was done under Olympus Inverted Fluorescence Microscope CKX53, Japan. Pure drugs (INH and RIF) ruptured the RBC's and distorted RBC's shape. It led to increased toxicity in blood while drug loaded co-polymeric micelles showed very less toxicity because of polymer (HPMA) which is more biocompatible can be seen **(**Fig. 6b**)**.

Cytotoxicity Studies, Microscopy and Anti-TB Assay

MABA test was performed for determination of MIC value of INH, RIF and PMRI micelles. MABA is a quick as well as efficient method in comparison to others (24). After 6 days of incubation of *M. tuberculosis*, colorimetric test results were noted for all strains. Alamar blue dye was added to the column B11 and incubated for 24 h, growth of *M. tuberculosis* was confirmed by change in colour from blue to pink. Alamar blue dye was added to rest of the all wells, plates were incubated for another 24 h and results were analysed **(**Fig. 7a**)**. MIC of INH and RIF was recorded 0.1 ± 0.02 and 0.5 ± 0.04 µg/mL respectively for sensitive strain. The MIC value of prepared formulation (PMRI) was observed to be 0.0625 ± 0.02 µg/mL for sensitive and 0.50 ± 0.08 µg/mL for resistant strain ($p < 0.0001$, sensitive MIC *Vs* resistant MIC in MABA) i.e. less than the pure drugs. In sensitive, PMRI was 2 folds more effective than pure INH and 8 folds than pure RIF, while in resistant, PMRI was 4 folds more effective than pure INH and 2 folds than pure RIF and the comparison shown in Fig. 7b. The obtained results were further analysed with the help of microscopic examination of *M. tuberculosis* at treated MIC of pure drugs (INH and RIF) as well as HPI and PMRI by fluorescence microscope (Olympus Inverted Fluorescence Microscope CKX53, Japan). The results showed that the growth of *M. tuberculosis* was inhibited by the HPI and PMRI significantly **(**Fig. 7c**)** in comparison to pure drugs because of polymeric micellar nanocarrier.

The microscopic results clearly showed that the prepared formulations were more effective in comparison to pure anti TB drugs **(**Fig. 7d**)**. It was clearly observed from the cytotoxicity assay that potency of micelles were higher than pure INH and RIF. This result confirmed that drug delivery through multidrug therapy will be beneficial along with reduce dosing frequency for patient compliance. Hence, it can be proved that co-delivery of drugs through same polymeric micelles can be an effective strategy to deliver anti-TB drugs.

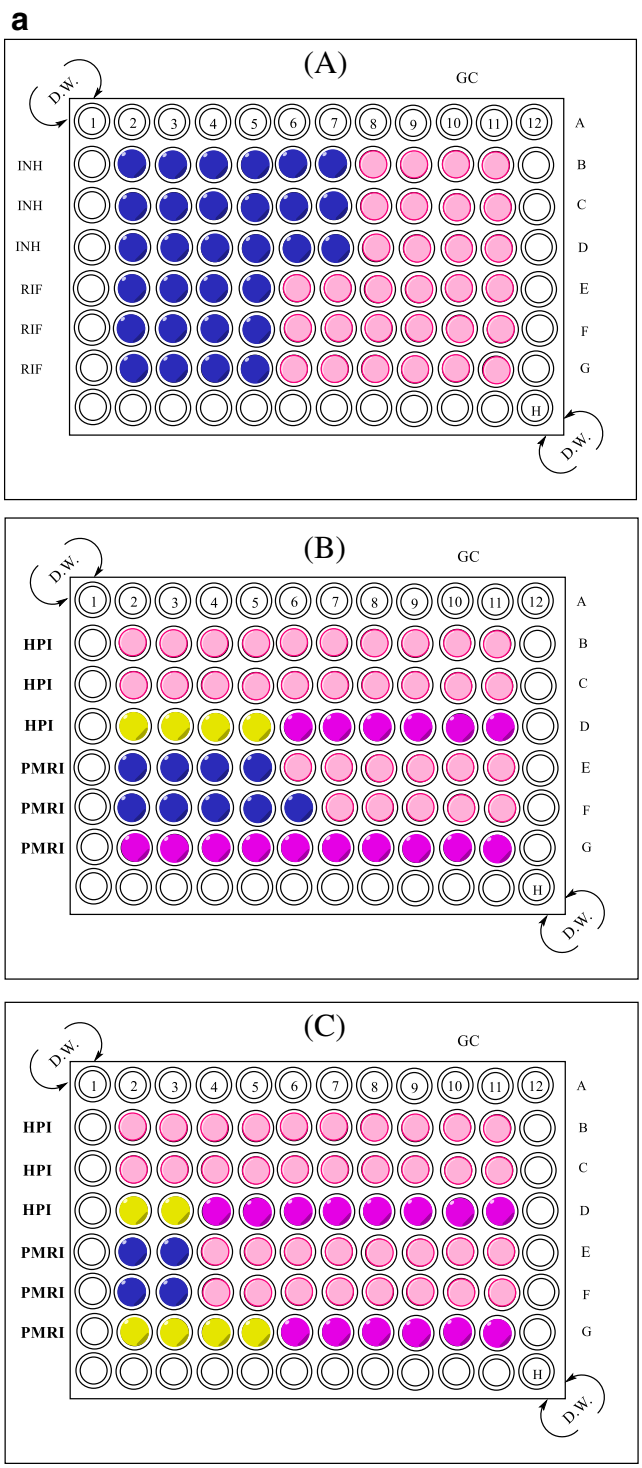


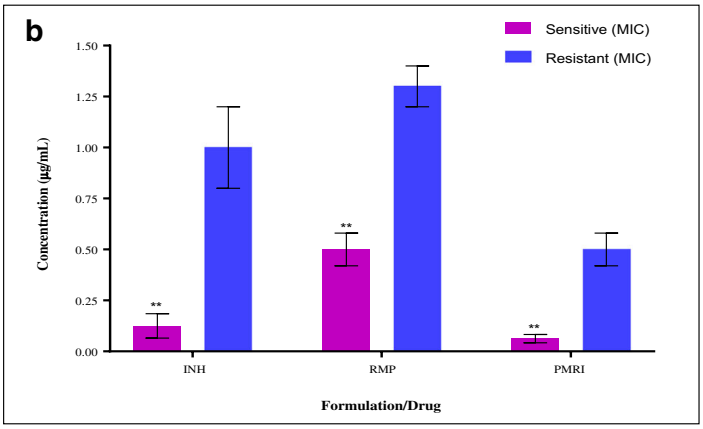
Fig. 7 **a** Cytotoxicity studies for determination of MIC value of drugs and formulation by MABA (Microplate-Based Alamar Blue Assay) *A* effect of pure INH & pure RIF *B* Effect of HPI & PMRI on Sensitive strain & *C* Effect of HPI & PMRI on resistant strain. [HPI: HPMA-PLA-INH conjugates, PMRI: Rifampicin loaded HPMA-PLA-INH co-polymeric micelles]. **b** Minimum inhibitory concentration of INH, RIF, PMRI against *A* sensitive *M. tuberculosis* (*purple*); *B* resistant *M. tuberculosis* (*blue*). Values represents mean ± SD ($n = 3$). [INH: Isoniazid, RIF: Rifampicin, PMRI: Rifampicin loaded HPMA-PLA-INH co-polymeric micelles]. **c** Microscopic study of cytotoxicity studies of drugs and formulation [RIF: Pure Rifampicin, INH: Pure Isoniazid, HPI: HPMA-PLA-INH conjugates, PMRI: Rifampicin loaded HPMA-PLA-INH co-polymeric micelles]. The number of *M. tuberculosis* decreases as the affectivity increases from left to right. **d** Microscopic images of *M. TUBERCULOSIS* at different concentration of formulation as well as pure drugs at 40X. (A) Growth of *Mycobacterium tuberculosis*. *B* pure INH treated. *C* & *D* pure RIF treated and (E) & (F) PMRI treated. [INH: Pure Isoniazid, RIF: Pure Rifampicin, PMRI: Rifampicin loaded HPMA-PLA-INH co-polymeric micelles].

CONCLUSIONS

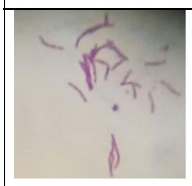
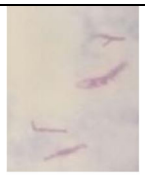
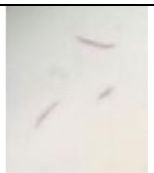
RIF loaded HPMA-PLA-INH co-polymeric micelles were successfully synthesized and characterized. In the present work, we aimed to enhance the effectiveness of INH and RIF with the help of polymeric micelles as a nanocarrier. The polymeric micelles are a novel drug carrier. In present study, HPMA acted as a hydrophilic shell and PLA as a hydrophobic core. After conjugation of HPMA-PLA, INH was further conjugated to develop HPMA-PLA-INH conjugate. RIF is a hydrophobic drug, because of the higher content of PLA in co-polymer, RIF was entrapped in micelles. RIF was easily loaded in the hydrophobic core due to hydrophobic interaction. The micellar size appears as the most crucial property. The formation of co-polymeric micelles enhances the effects of drugs, enhances the solubility of drug and counter the drug resistance developed by anti-TB drugs. Co-polymeric micelles enhanced the solubility of RIF and in addition reduced the overall hemolytic toxicity.

In vitro effectivity testing against strains of *M. tuberculosis* is a strenuous job. The culture of this gram negative (−ve) bacteria necessitates special equipment and facility and that is the reason the reported studies were conducted at the National Japanese Leprosy Mission for Asia (JALMA) Institute of Leprosy and Other Mycobacterial Diseases, Agra, India. The institute is among one of few institute designated by Government of India to study the genetic framework of *M. tuberculosis* as well as other diseases. The assay revealed that the effect of INH and RIF was enhanced when tested with the co-polymeric micelles. The prepared formulation has resulted into minimal hemolytic toxicity. The release data shows that the breaking of hydrazone bond leads to release of INH from co-polymeric micelles, while loaded RIF is released as a burst from co-polymeric micelles. RIF release was fast in the initial stage and slowed down at later time. The rate of release was reduced because the PLA content was increased. This approach of co-delivery was novel and promising and has immense potential of safe and effective delivery of multiple anti-TB drugs.

Multiple drug delivery is prerequisite in the mycobacterium induced infections such as pulmonary tuberculosis as well as others. The available chemotherapy is unpopular and is not



c

Growth Control	At MIC			
	RIF	INH	HPI	PMRI
				

d

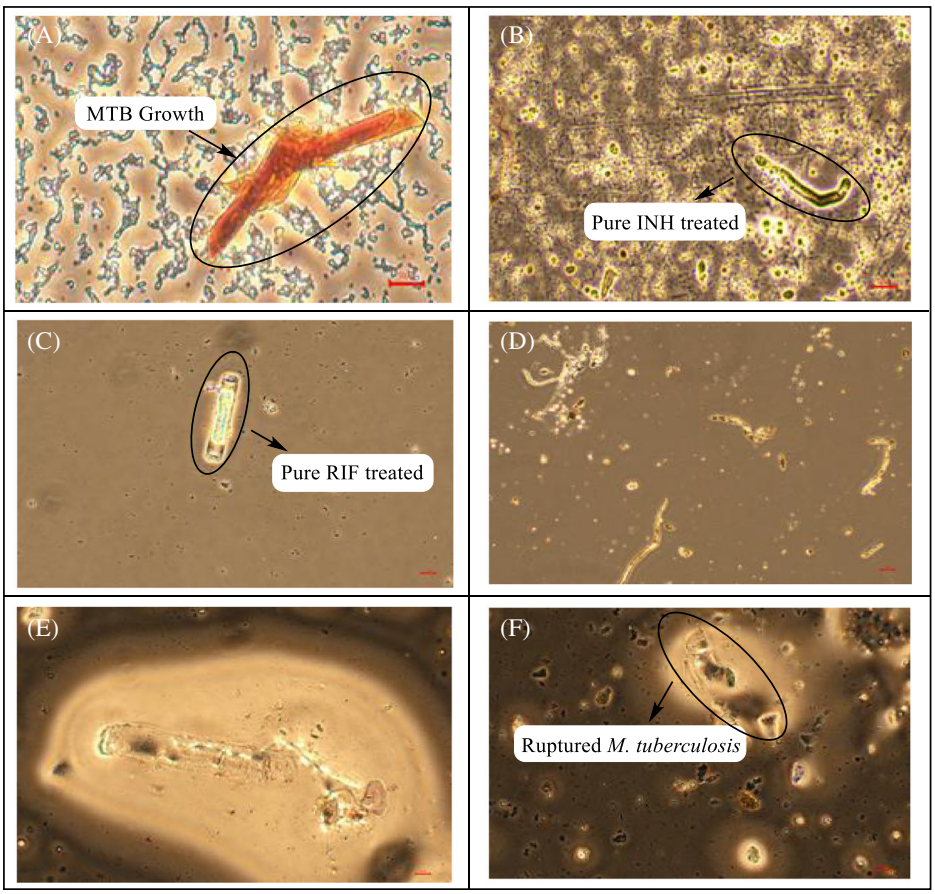


Fig. 7 continued.

patient friendly due to its long therapy duration as well as separate administration of each drug daily. It is even more difficult to patient to remember the dosing. Utilizing the key advantages of nanocarriers such as polymeric micelles we can ameliorate the shortcomings in the existing chemotherapy against *M. tuberculosis*. The present study is a preliminary attempt which has proved that combining two anti TB drugs can be beneficial and leads to better results against sensitive as well as resistant strains of *M. tuberculosis*. Though the study reports only *in vitro* study but the observed results clearly indicates its novelty and remarkable approach to deliver duo drugs against *M. tuberculosis*. It also reports equally that the use of HPMA can-not be limited only against cancers rather it has immense potential as drug delivery carrier pertaining to other disorders as well.

ACKNOWLEDGMENTS AND DISCLOSURES. The authors would like to acknowledge, the financial support received from Department of Science and Technology to Dr. Umesh Gupta in the form of Rajasthan DST R & D project [File no. 7 (3) S & T/R & D/2016/6074]. The authors like to acknowledge the, Kwality Pharmaceutical Limited, Amritsar, Punjab, India for providing rifampicin and isoniazid as gift samples. The authors would also like to acknowledge the M/s Evonic Industries, Germany for providing gift sample of PLA.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare no competing financial interest.

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