

Targeted Delivery of Paromomycin to Leishmania Infected Macrophage by Hemoglobin Tagged Nanocarrier

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Abstract

Purpose: After the extensive resistance of the first line of antimonial drugs, paromomycin, the broad-spectrum antibiotic has become the treatment of choice against visceral leishmaniasis, the fatal tropical disease. However, unfavorable distribution in the intracellular compartment of macrophage, prolonged parenteral administration procedure and toxicity limit the use of paromomycin. Lack of availability of specific delivery system also makes this drug unsafe. Thus, a specific and nontoxic formulation of paromomycin is an urgent need.

Materials and method: A chitosan-chondroitin sulfate based nano-formulation has been developed and hemoglobin has been tagged on the surface of the delivery system for the specific carriage of paromomycin to the leishmania infected macrophage taking the advantage of Leishmania being highly auxotrophic for heme.

Results: There has been a significant improvement in the toxicity profile with lowered LD50 value of nanoformulated paromomycin (75 μ M) compared to the direct administration (130 μ M).

Conclusion: A cheap, biodegradable, nontoxic and specific nano-carrier has been introduced for specific delivery of paromomycin to infected macrophages

Keywords: Visceral leishmaniasis; Paromomycin; Nano-formulation; Chitosan; Chondroitin sulfate; Hemoglobin; Drug delivery

Introduction

Visceral leishmaniasis (VL) or kala-azar, caused by the parasite *Leishmania donovani* (LD) and regarded as one of the most fatal tropical disease if left untreated, is endemic in Indian subcontinent and Africa [1,2]. Around the globe, approximately 12 million people in 98 countries are suffering from different form of leishmaniasis with 2 million of new infection each year [3-5]. Among the admissible first-line drugs, pentavalent antimonials (Sb⁵⁺) and amphotericin B are toxic, requiring parenteral administration, prolonged treatment duration, and hospitalization. In addition, over 60-70% cases of resistance to pentavalent antimonials in the Indian endemic regions have posed a real challenge before the clinical management of VL [6]. Among the other, miltefosine is the only oral drug, however, prolong treatment time, gastrointestinal-hepatotoxicity and resistance limit its use [7]. Paromomycin (PMM), the aminoglycoside antibiotic produced by *Streptomyces rimosus* var. *paromomycinus* is one of the most promising antileishmanial drugs that showed an excellent performance in phase IV trial with favorable safety profile and over 94% cure rate [8]. Susceptibility of both antimony-resistant and -sensitive LD isolates to PMM is one of the crucial advantages of its use in the endemic region of Indian subcontinent [9]. However, because of high molecular mass (615.6 g/mol) and hydrophilicity of PMM, the effective concentration remains to be very low in the intracellular compartment of macrophages, nesting the LD amastigotes. Among other disadvantages, dose-dependent adverse side effects including injection site toxicity, ototoxicity, nephrotoxicity, hepatic toxicity and tetany are the issues often reported [7,10]. To address these problems, biodegradable mixed polymeric nanoparticles of chitosan (Ch)-chondroitin sulfate (Chs) have been introduced as a carrier for PMM [11-13]. Leishmania parasite is auxotrophic for heme, which is an essential nutrient for them for the growth and various metabolic pathways, and they have to manage it from the host. Consequently, VL patients often suffer from one of the most severe forms of anemia. Thus, Leishmania

amastigotes residing inside the macrophages induce various molecular machineries for effective endocytosis of Hb by its host [14]. Therefore, heme acquisition mechanisms collectively, can be a very effective drug target against LD amastigotes inside the macrophage [15]. LD employs the over expression of diverse Hb-binding receptors like high affinity receptor (HbR), CD163, Heme Carrier Protein-1 (HCP-1), ferrous iron transporter (DMT-1) and CD91 in infected macrophages for Hb internalization and its degradation and heme assimilation [15-21]. Therefore, in our design of Cs-Chs nanocarrier for delivery of PMM specifically to the LD infected macrophage, the Hb uptake mechanisms were targeted as the drug delivery route by decorating the surface of Cs-Chs nanoparticles by Hb. Due to the targeted delivery of PMM via Hb-uptaking mechanisms, higher local drug concentration is envisaged around of infected macrophages. Further, this new formulation of PMM would be cost efficient and less toxic because of the use of natural, biodegradable constituents like chitosan, chondroitin sulfate and Hb [22]. To our knowledge, this is the first biodegradable nanocarrier of PMM that delivers the drug specifically into the infected macrophage via the Hb-assimilating molecular pathways, with lesser toxicity and side effects.

Materials and Method

Chitosan (MW 60,000-120,000), Chondroitin sulfate, TPP (Sodium tri-poly phosphate, MW 367.86), Hemoglobin, MES (2-morpholino-

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Received: December 21, 2015; **Accepted:** January 12, 2016; **Published:** January 16, 2016

Citation: Kumar P, Bose PP (2016) Targeted Delivery of Paromomycin to Leishmania Infected Macrophage by Hemoglobin Tagged Nanocarrier. J App Pharm 8: 212. doi:10.4172/1920-4159.1000212

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ethansulfonsaure monohydrate buffer, Dimethyl sulfoxide (DMSO; analytical grade), MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide), Trypan blue (Himedia), phosphate buffer saline, cell culture media (RPMI 1640, M 199), EDC (1-ethyl-3-[3-dimethyl amino propyl] carbo-diimide), NHS (N-hydroxy succinimide), Ficoll and paromomycin sulfate (PMM) were purchased from Sigma-Aldrich. Acetic acid, methanol and Giemsa staining kit were purchased from Himedia Laboratories. *Leishmania donovani* (MHOM/IN/83/AG83) and Human PBMC were used for various *in vitro* analyses.

Preparation of chitosan-choindroitinsulfate nanoparticles (Cs-Chs NP) and paromomycin loaded chitosan-chondroitin sulfate nanoparticles (Cs-ChsPMM NP)

Nanoparticle used in this study was prepared according to the Polyelectrolyte Complexation Techniques (PEC). To prepare the NP, 5 mg of chitosan (Cs) was dissolved in 2 ml of 2% (v/v) acetic acid. 20 mg Chondroitin sulfate (ChS) was dissolved in 2 ml of deionized water to make a stock and serially diluted to obtain the solutions with concentrations 10, 5, 1.25 and 0.62 mg/ml (w/v). The above solutions were mixed together such that the Cs/ChS (w/w) reaching theoretical ratios of 1:60, 1:48, 1:24 and 1:12, respectively at pH ~ 6.

To prepare Cs-Chs-PMM nanoparticle, PMM of concentration 500 µg/ml was dissolved in distilled water, and added to the Cs, ChS mixture of different ratios followed by the addition of 1 ml of 1 mg/ml sodium tripolyphosphate (TPP) solution to each of the mixture (2 ml).

Briefly, (for 1:60 Cs-Chs NP), in a tube 50 µl of chitosan solution (2.5 mg/ml dissolved in 2% acetic acid), 750 µl of chondroitin sulfate (10 mg/ml dissolved in water), 50 µl of PMM (as the mentioned concentration as above) and 50 µl of TPP was added and the mixture was sonicated for half an hour with interval after each 5 mins. The mixture was allowed to stand for 30 min in dark and washed with distilled water under centrifugation at 10000 RPM for three times to remove the residual unloaded PMM. Finally the nanoparticles were resuspended in double distilled water and kept at 4°C for future use.

Conjugation of hemoglobin on the surface of nanoparticles

The different concentration of Hb solutions were prepared from a Hb stock of 10 mg/ml, diluting in 1 mM MES (pH 6.5) buffer such that the solutions contain 250, 500, 750 µg/ml of Hb. Activation of Hb was done by adding 3.75 µl of 1% EDC (in water) to the hemoglobin solution. After gentle mixing for 2-3 minutes 3.75 µl of 1% NHS (in DMSO) was added and allowed to stand for 15 minutes. 200 µl of concentrations of Cs-ChS-PMM nanoparticles (250 µg/ml) were then added to the pre-activated hemoglobin solution. Now mixture was mixed with the help of rotospin in 25 RPM for 3 hours at dark and room temperature.

Drug entrapment assay

For synthesizing 1:24 Cs-Chs Np, 500 µl of chitosan solution (2.5 mg/ml dissolved in 2% acetic acid) and 300 µl of chondroitin sulfate (10 mg/ml dissolved in water) solution were added to 500 µl of Fmoc-PMM (1 mg/ml in water) with brief stirring followed by the addition of 500 µl of TPP. Then the mixture was sonicated for 30 sec. Now the mixture was allowed to stand for 1 hr in dark and was centrifuged at 10000 RPM after each 5 mins. At each interval fluorescence of the supernatant was measured at 315 nm up on excitation at 260 nm.

Drug release assay

Since PMM is not an UV active molecule therefore, to study its release from the Cs-Chs nanoparticle we first synthesized the Fmoc-PMM by solution phase reaction. Briefly, 4 mM solution of Fmoc-Cl

(10.3 mg) in acetonitrile (10 ml) was mixed with 10 ml of 0.5 mM solution of PMM in borate buffer (pH 8.1, 0.5 M) under stirring for 15 min in dark. After completion of the reaction, the mixture was extracted with ethylacetate (thrice) and thereafter the ethylacetate layer was evaporated to yield Fmoc-PMM, which was used for drug release study.

Same procedure as above was followed to entrap Fmoc-PMM in Cs-Chs NP (1:24). The concentration of the drug coming out in the solution from Cs-Chs nanocarrier was determined by measurement of fluorescence of released Fmoc-PMM from Cs-Chs nanoparticle and was assessed for 16 hrs after the releasing solvent (10:10:80 Methanol:DMSO:Water) was added to the washed drug loaded NPs (200 µl suspension of 1 mg/ml NPs were washed and subjected to release assay). After each time intervals nanoparticles were centrifuged at 10000 RPM for 10 minutes and then supernatant solution was taken and fluorescence was measured at 315 nm in spectrofluorimeter under an excitation at 260 nm.

Transmission electronic microscopy

Aggregation state of the PMM loaded nanoparticles were determined by transmission electron microscopy (TEM; JEOL 2010) under an accelerating voltage of 100 kV. Cs-Chs-Hb-PMM nanoparticle was suspended in human sera at concentration of 1 mg/ml and kept at 25°C for 48 hrs followed by washing with double distilled water up on centrifugation at 10000 RPM for 10 min. Next the nanoparticles were resuspended in water and were dropped on carbon coated copper grids (300 mesh). Then the grids containing the drop of sample solution were dried under vacuum.

Biological assays

***L. donovani* promastigote culture and maintenance:** Reference strain of *L. donovani* MHOM/IN/83/AG83 was used in this study. The *L. donovani* promastigotes culture was maintained in M199 complete medium containing 10% heat inactivated fetal bovine serum, pH 7.2 to 7.4 at 24 ± 1°C.

Treatment of *L. donovani* promastigotes with Cs-Chs-PMM nanoparticles to evaluate its antileishmanial efficacy: Antileishmanial efficacy of Cs-Chs PMM nanoparticles on the growth of promastigotes was evaluated. PMM loaded Nanoparticles concentration were dispensed in 96 well culture plate in triplicate series and final concentration was taken 250, 200, 180, 150, 140, 120, 100, 80, 50, 10 µg/ml. Similarly for positive control of PMM was taken in triplicate series. It was then taken at final concentration of 250, 200, 180, 150, 140, 120, 100, 80, 50, 10 µg/ml. Finally 50 µl of 2 × 10⁶ *L. donovani* promastigote/ml were dispersed in each well to get final concentration of 1 × 10⁶ promastigotes/ml. Cultures were further incubated at 24 ± 1°C in BOD incubation. Cell count was performed after 48 hours of incubation and subjected to morphological analysis under microscope using 0.1 mm Nauebauer chamber (Fien optic, JENA, Germany).

Development of *in vitro* amastigote model for drug efficacy evaluation: Human peripheral blood mononuclear cells (PBMC) were isolated with the help of gradient centrifugation using Ficoll-Hypaque (Histopaque), and after 2 hours incubation at 37°C, in a CO₂ incubator, the monocyte-derived macrophages were isolated by adherence method. Cells (1 × 10⁶/ml) were dispensed in 96 well culture plates for *L. donovani* infection. Finally, macrophages were maintained in RPMI 1640 supplemented with 10% FBS, 50U penicillin/ml and 50 µg streptomycin/ml at 37°C, in a CO₂ incubator providing 5% CO₂ and 95% humidity. Late log phase *L. donovani* promastigotes were introduced in 1:10 ratio (Macrophage: *L. donovani*). The culture was

further incubated at 37°C, in a CO₂ incubator for overnight. To remove non-phagocytosed promastigotes, culture plate was washed with RPMI medium. Hb-conjugated polymeric nanoparticles were assessed by cell culture was kept at 25°C in BOD incubator to get promastigote form of *L. donovani* for confirmation of presence and survival of the parasite after treatment as well as in control.

Cytotoxicity study

In vitro cytotoxicity of formulated PMM on human MNC by MTT: Cytotoxicity of the new formulations was evaluated by Mitochondrial dehydrogenase based 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). This assay was performed using an MTT-based *in vitro* toxicity assay Kit (sigma, USA). Briefly, monocyte-derived macrophages were cultured either with or without nanoparticle component or controls at different concentrations; negative control (without drug treatment) and positive control (treatment with PMM drug without NP) in phenol red free RPMI 1640. After 48 hrs treatments, cultures were supplemented with MTT solution (needed 10% according to medium volume) and incubated in BOD incubator at 25 ± 1°C for 2 hours. Then cells were supplemented with 220 µl MTT solubilisation solution that was supplied with the Kit. Vortex was performed before reading to dissolve formazan crystals. The samples were subsequently analysed with a plate reader at a wavelength of 570 nm.

In vitro cytotoxicity of formulated PMM on HeLa cell by trypan blue exclusion method: Various chitosan nanoparticles of PMM along with the free drug were studied for *in vitro* cytotoxicity using trypan blue dye exclusion method. HeLa cell line was seeded in 24-well plates. Three wells for each formulations and concentrations were seeded. Then, the cells were incubated at 37°C in presence of 5% CO₂. After 16 h the medium was replaced by fresh one and different nanoformulations along with free drug were added, followed by incubation for 48 hrs. After incubation, 0.1 ml trypan blue was added and number of dead cells determined by using haemocytometer.

Results and Discussion

Physicochemical characterization of nanoparticle based drug delivery system

To serve as an effective carrier and release of drug within biological system, the nanoparticles must be non-aggregating with retention of its size-shape for long time in circulatory system. Thus, the morphological parameters of the nanoparticle (Cs-Chs-Hb-PMM) were compared by storing them in human sera (1 mg/ml) for 48 hrs at 25°C. Under TEM study, the nanoparticle appeared as discrete, dense spherical particles of approximately 200 nm (Figure 1A). From the detailed study of morphology as demonstrated in the TEM micrograph, it was evident that no aggregation was triggered in the nanoparticles up on storage in human serum for 48 hrs. Thus, Cs-Chs-Hb-PMM nanoparticles were found to be non-aggregating, retaining its structural integrity in human sera which was essential for long circulation time *in vivo*.

It is worth mentioning that due to non-fluorogenic nature of PMM, the entrapment and release studies were performed with its Fmoc-derivative, which showed a stable emission at 315 nm upon excited at 260 nm.

In the drug entrapment study Cs-Chs nanoparticle was synthesized in presence of Fmoc-PMM by PEC method. The entrapped amount of Fmoc-PMM in Cs-Chs nanoparticle was assessed by measuring the residual amount of the drug in the supernatant by fluorescence emission at 315 nm. As result suggested (Figure 1B) that there was a gradual decrease in the entrapment with a lag phase of initial 30 mins

while approximately 30% drug was found to be loaded slowly. During following 25 mins loading was sharply increased up to a maximum of 87% with no further increase.

In the release study, Fmoc-PMM released from drug loaded nanoparticles was estimated in MeOH:DMSO:Water 10:10:80, and the estimation was carried out by measuring fluorescence of Fmoc-PMM on spectrofluorimeter before and after the addition of nanoparticles in the releasing solvent (MeOH:DMSO:Water 10:10:80). Release experiment was carried out during 16 hrs after the addition of releasing solvent to the drug loaded NPs.

According to the release profile (Figure 1C), at 16 hrs nanoparticles showed the percentages of drug release to be ~98% for Cs-Chs-NPs (Cs-Chs 1:24) loaded with 250 µg/ml PMM. It was evident that at initial 3 hrs only 40 % drug was released but after 9 hrs more than 90% drug got released from Cs-Chs-NPs loaded with PMM. Therefore, for sustained and slow release, this nanoparticle formulation was chosen for our further drug delivery experiment with LD promastigotes and intracellular amastigotes in infected macrophages. The delivery of the drug in soluble form, inside the infected macrophage is highly envisaged by this formulation as the parasitophorous vacuoles of the host macrophage where the amastigotes are nested, maintain very low pH, where polymeric chitosan is expected to get disrupted and deliver PMM to the amastigotes residing there.

Treatment of *L. donovani* promastigotes with Cs-Chs-Hb-PMM nanoparticles to evaluate its anti-leishmanial efficacy

The effect of PMM loaded nanoparticle (Cs-Chs-Hb-PMM) on survival of *L. donovani* promastigotes were determined in comparison with untreated control after 24 and 48 hrs of treatment (as negative control) and as positive control direct PMM treatment was administered (Figure 2). IC₅₀ value of PMM loaded nanoparticles (Cs-Chs-PMM) was evaluated and compared with the normal PMM; and dose based effect was shown in Figure 2C; the Cs-Chs-PMM formulation was found to have higher efficacy than the normal PMM. On our study, IC₅₀ of normal PMM was determined to be 135 µM, while Cs-Chs-Hb-PMM nanoparticle showed the IC₅₀ to be 80 µM (Figure 2). It indicated the higher efficacy of our nanoformulated drug against LD promastigotes as compared normal dose of the same drug. In Figures 2A and 2B, population density of PMM treated and Cs-Chs-Hb-PMM treated promastigotes were compared. There was a significant drop in the population of promastigotes were observed under the treatment of Cs-Chs-Hb-PMM compared to the direct dose of PMM. The motility of promastigotes underwent Cs-Chs-Hb-PMM treatment was found to be significantly less with a change in morphology as compared to the promastigotes had direct PMM treatment.

Leishmanicidal effect of nanoparticles on amastigotes: Specific delivery at infected macrophage

To assess the effect of specific delivery of PMM to the infected macrophage, and to compare the effect of PMM and its two formulations (Cs-Chs PMM and Cs-Chs-Hb PMM) on the LD amastigotes residing as intracellular parasites in side macrophages (Figure 3A), the promastigotes developed form residual amastigotes after different treatments were enumerated microscopically. Under BOD incubation, LD promastigotes were emerged from amastigotes surviving the treatments of PMM and its different formulations on infected macrophages and were subsequently evaluated microscopically as described earlier. LD₅₀ of PMM, Cs-Chs PMM, and Cs-Chs-Hb PMM formulations were found to be 130 ± 2, 100 ± 5, and 75 ± 5 µM

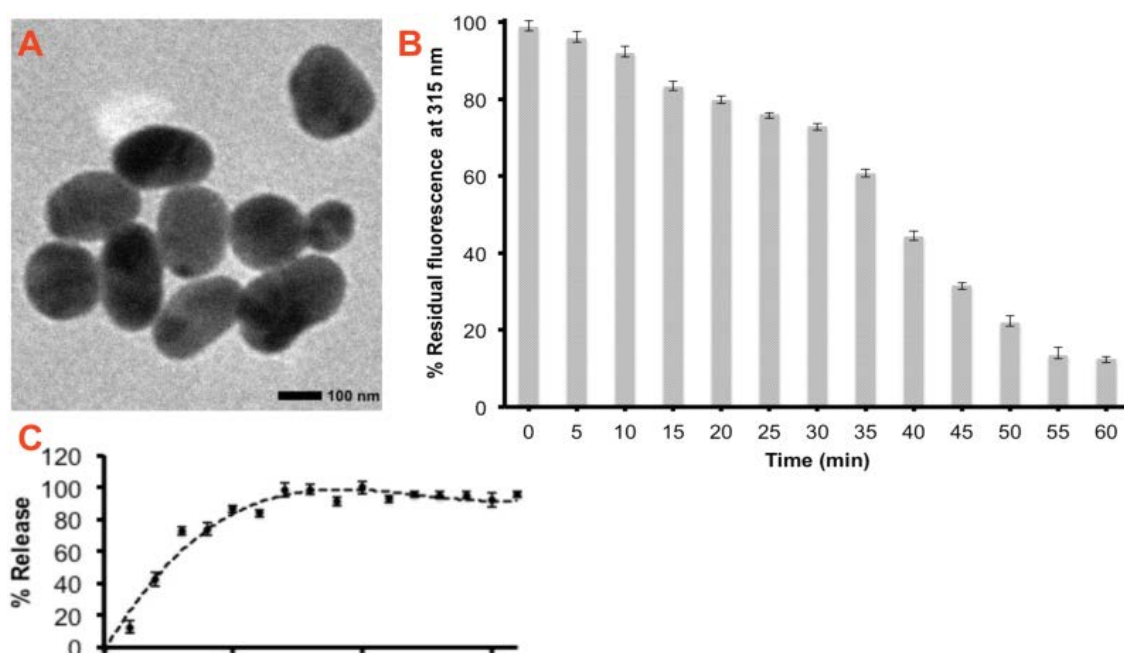


Figure 1: A) Transmission electron microscope experiment represented the size and polydispersity of nanoparticles; where the nanoparticle Cs: ChS ratio 1:24 loaded with PMM drug and conjugated with Hb was characterized having radius around 200 nm. B) Drug entrapment assay was performed by measuring the fluorescence at 315 nm for the residual Fmoc-PMM in the supernatant in presence of Cs-Chs Np for 1 hr. C) Drug release assay was performed in 10:10:80 Methanol:DMSO:Water as solvent for NPs with 250 µg/ml loaded drug concentrations; the release of PMM was recorded for 16 hrs. At 16 hrs ~ 98% drug was released.

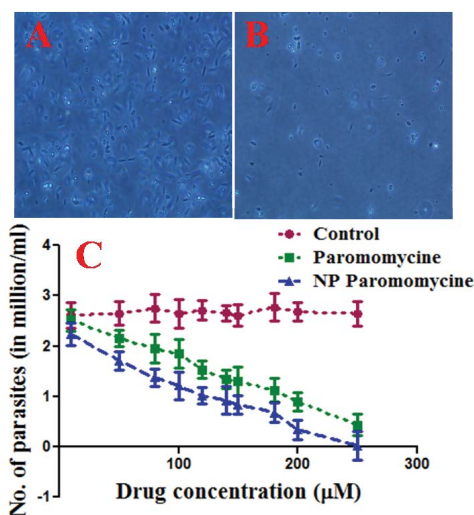


Figure 2: Pictorial presentation of the activity of NP PMM formulation on promastigotes; A) Cs-Chs-Hb NP without drug as control. The cells were healthy, motile with high population density. B) Under the treatment of Cs-Chs Hb-PMM reduced number of cells with changed cell morphology and less motility were observed. C) Normal PMM with IC₅₀ 135 µM at 48 hrs. Cs-Chs Hb-PMM with IC₅₀ was found to be 80 µM at 48 hrs.

respectively (Figure 3B). This experiment confirmed the lower LD₅₀ concentration of Cs-Chs-Hb PMM than that of Cs-Chs-PMM or direct PMM treatment that indicated the enhanced specificity of Hb guided Cs-Chs-PMM nanoformulation which probably enhance the local drug concentration around the infected macrophages and became more

efficient for acting against intracellular amastigotes. Whereas for Cs-Chs PMM nanoformulation the delivery vehicles were not specifically targeted to the infected macrophage surface only, therefore, higher LD₅₀ concentration was recorded. Probably Cs-Chs-PMM attacked both the infected and noninfected macrophage because of the lack of specific targeting molecule like Hb. Thus, this specific delivery system decreased the LD₅₀ dose of PMM as compared to pure PMM and non-targeted nanoformulation of the same.

Cell toxicity study of different forms of nanoformulations

LD₅₀ concentrations for amastigotes were found to be nontoxic for both Ch-Chs-PPM and Cs-Chs-Hb-PPM for human mononuclear cells (MNCs) and HeLa cells in comparison to pure PMM as MTT and trypan blue assay revealed respectively (Figures 4A and 4B) on the same cells. Toxic effect of free PMM on HeLa cells were evident in the significantly altered morphology of the dead cells post treatment with free PMM compared to the non-treated control cells (Figures 4C and 4D). Therefore, it is envisaged that use of this nanoformulations would produce lesser overall toxicity than does the free PMM.

The hypothesis of specific targeting of infected macrophages has been elucidated in Scheme 1. The nanoparticle was made up of biodegradable polymers of chitosan and chondroitin sulphate and the guide molecule, Hb was covalently tagged on the surface of the nanoparticles. These made the Cs-Chs-Hb more biocompatible and less toxic. LD is auxochromic to heme, like other hemoflagellates and thus, it has developed a robust set of Hb-scavenging molecular mechanisms to assimilate Hb from the host. In our current endeavor, we targeted these over expressed Hb-internalizing receptors on cell surface of infected macrophages as the routes to deliver the drug specifically from the Ch-Chs-Hb-PMM nanocarrier to the amastigotes nested inside the macrophage. This nanocarrier comprised of biodegradable polymers

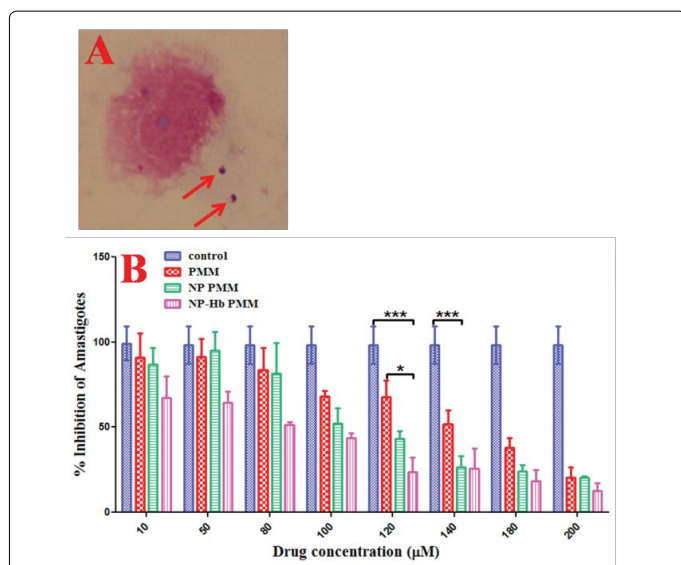
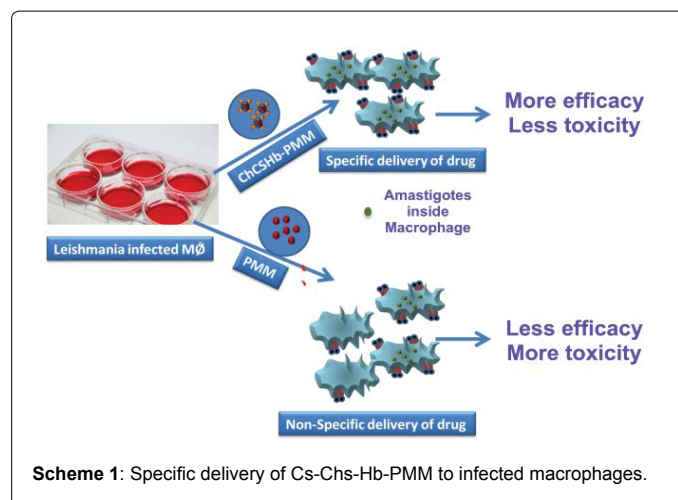


Figure 3: A) Infection of leishmania to human healthy macrophages and the production of amastigote was established by introducing promastigote to the culture of macrophage at 1:10 ration of macrophage:promastigote. Giemsa stain was used to stain the amastigotes and macrophage nucleus to count the amastigotes under microscope at 100X; where red lines indicated the amastigote cells. **B)** Graph indicated the percentage inhibition of amastigotes at 48 hrs after treatment of infected macrophages by normal PMM, Cs-Chs PMM and Cs-Chs-Hb PMM; one untreated control was also taken. A significant decrease in LD50 concentration was recorded for Cs-Chs-Hb PMM nanoformulation (75 µM) as compared to Cs-Chs PMM (100 µM) and PMM (130 µM). Samples were analyzed by two ways Anova with statistical differences indicated as follows: p<0.001, *** for control vs. Cs-Chs PMM (140 µM), p<0.001, *** for control vs. Cs-Chs Hb-PMM (120 µM) and p<0.05,* for PMM vs. Cs-Chs Hb-PMM (120 µM).



Scheme 1: Specific delivery of Cs-Chs-Hb-PMM to infected macrophages.

has shown their nonaggregating nature upon storage in serum (Figure 1A) which ensured the putative stability of this delivery vehicle during their circulation within human system. This biodegradable, specific and cheap PMM loaded nanocarrier lowered the IC50 for promastigotes and LD50 for intracellular amastigotes compared to the classical direct PMM treatment. The toxicity profile of Cs-Chs-Hb-PMM was shown to be favorable compared to that of the LD50 doses of pure PMM and non-target specific Cs-Chs PMM formulation. Thus, this new nanoformulation of PMM can find its uses in the clinical management of VL, especially in the treatment failure cases of antimonial drugs for its better efficacy, lesser toxicity and target specificity.

Conclusion

There has been an extensive impetus in the drug development research against the parasitic diseases including leishmaniasis since the last two decades. Target based drug development has produced some important antileishmanial compounds. However, toxic side effects, unfavorable physico-chemical properties and lack of target specificity often limit the use of many of these promising anti-leishmanial drugs. Nanoformulation has been one of the most prominent strategies for effective and specific delivery of drugs. In our current endeavor, we have used biodegradable nanoparticle made up of natural polymers like chitosan and chondroitin sulfate. The delivery of the drug (PMM) encapsulated in the nanoparticle was highly envisaged because of the plausible degradation of the Cs-Chs copolymer inside the highly acidic intracellular environment of macrophage where the LD amastigotes were inhabited. To enhance the specificity of the nanoformulation, it was functionalized with Hb, which made the nanoformulation target specific towards the Hb-internalizing mechanisms of LD infected macrophages. Our study established that the current novel Hb-coated Cs-Chs nanoformulation could be used as a carrier of choice for targeted delivery of other anti-leishmanial drugs with enhanced efficacy and lowered toxicity.

Acknowledgement

PPB thanks Department of Science and Technology (DST), India for Fast Track Young Scientist project (SB/FTP/CS-017/2013) and Bose Institute, Kolkata for research infrastructure. The authors acknowledge NIPER-Hajipur, Ministry of Chemicals and Fertilizers, Govt. of India. Authors declare no conflict of interest in this work.

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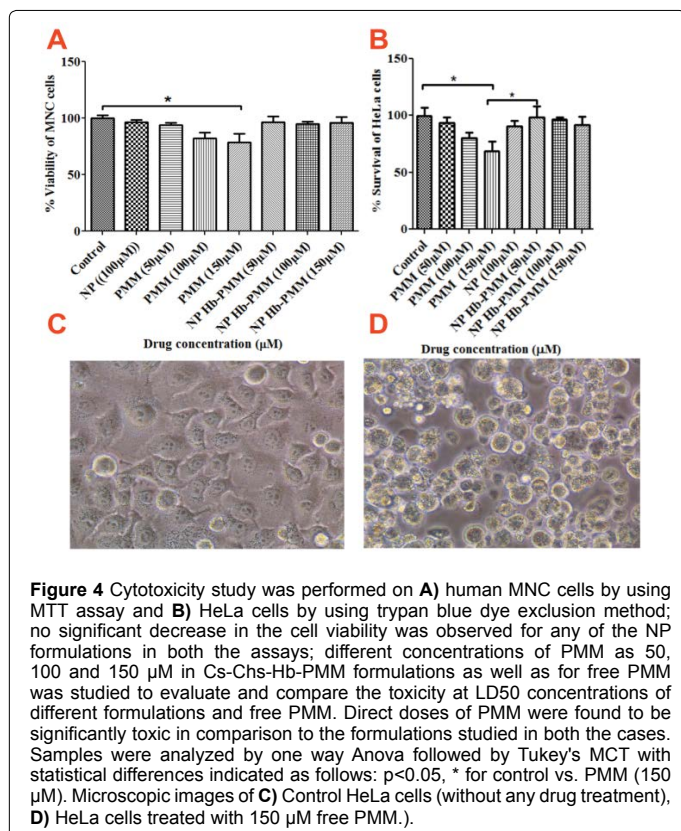


Figure 4 Cytotoxicity study was performed on **A)** human MNC cells by using MTT assay and **B)** HeLa cells by using trypan blue dye exclusion method; no significant decrease in the cell viability was observed for any of the NP formulations in both the assays; different concentrations of PMM as 50, 100 and 150 µM in Cs-Chs-Hb-PMM formulations as well as for free PMM was studied to evaluate and compare the toxicity at LD50 concentrations of different formulations and free PMM. Direct doses of PMM were found to be significantly toxic in comparison to the formulations studied in both the cases. Samples were analyzed by one way Anova followed by Tukey's MCT with statistical differences indicated as follows: p<0.05, * for control vs. PMM (150 µM). Microscopic images of **C)** Control HeLa cells (without any drug treatment), **D)** HeLa cells treated with 150 µM free PMM.).

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