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## TARGETED DELIVERY OF CATALASE TO MACROPHAGES BY USING SURFACE MODIFICATION OF BIODEGRADABLE NANOPARTICLE

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**ABSTRACT:** Reactive oxygen species (ROS) produced by activated macrophages play a major role in causing liver disorders. Small antioxidants and scavengers are protective only at very high concentrations and they poorly detoxify toxic ROS. Catalase (CAT) is an antioxidant enzyme that scavenges ROS and has great potential for treating liver disorders. In the present research work, gelatin nanoparticles bearing CAT were prepared using a two-step desolvation technique coupled with mannose and targeted in to liver to treat liver disorders associated with excess of ROS. It was characterized for shape, particle size, zeta potential, and percentage drug release and entrapment and confirmed by infrared spectroscopic studies. Experimental studies demonstrated that mannose Coupling enhanced their uptake into macrophages due to presence of mannose receptors in macrophages which increases their ability to scavenge ROS, produced by the activated macrophages.

**INTRODUCTION:** Reactive Oxygen Species (ROS) represents a variety of diverse species including superoxide anions ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radicals ( $HO\cdot$ ), singlet oxygen. Some of these species ( $HO\cdot$ ,  $O_2^-$ ) are known as radicals (molecules containing unpaired electrons) and are extremely unstable and involve in the pathogenesis of a number of diseases, including atherosclerosis, cancer and Alzheimer's disease, most liver diseases, including chronic viral hepatitis alcoholic hepatitis, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH) and chronic cholestasis <sup>1-3</sup>.

ROS induces cellular damage by oxidization of lipids, proteins and nucleic acids <sup>4</sup>. To encounter this, cells have antioxidant defense mechanisms against ROS, including ROS-scavenging enzymes <sup>5</sup>.

Catalase (CAT, EC 1.11.1.6) is an antioxidant defense enzyme that catalyzes the redox disproportionation of the toxic oxygen metabolite,  $H_2O_2$ , into dioxygen and water <sup>5</sup>. Catalase is an iron-containing antioxidant enzyme is primary found in peroxisomes. It is predominantly concentrated in liver and it detoxifies hydrogen peroxide by catalyzing a reaction between two hydrogen peroxide molecules, resulting in the production of water and oxygen.

The enzyme has been isolated from various sources, including bacteria and plant cells <sup>6-7</sup>. However, clinical trials with these enzymes have provided little therapeutic benefits, due to drug delivery problems, and delivery vehicles are greatly needed that can

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enhance their efficacy and it is known that CAT is rapidly eliminated from the circulation after intravenous bolus injection, which limits its therapeutic potential<sup>8</sup>.

To increase its therapeutic potential, targeted delivery systems for CAT have been developed involving direct modification, such as galactosylation, mannosylation, succinylation or polyethylene glycol conjugation<sup>9</sup>.

CAT has been successfully targeted to liver nonparenchymal cells by succinylation or mannosylation, which is a promising approach to prevent liver injury by reducing the ROS produced by macrophages and neutrophils infiltrating the organ<sup>10</sup>. There are so many carriers are available for macrophage targeting like liposomes<sup>11</sup> microspheres<sup>12</sup> and nanoparticles. As compared to other carrier systems, nanoparticles are known to have better stability and accumulation in macrophage rich organs, e.g., liver, spleen, and brain due to their preferential phagocytosis<sup>14</sup>.

Therefore, nanoparticles represent an attractive and promisable approach for the targeted delivery of antioxidant agents to macrophages. Macrophages are able to internalize particulate carriers through endocytosis and possess various surface receptors such as Fc, mannosyl, lectin and galactosyl<sup>15</sup> which is help them in the process of recognition and endocytosis of a particulate carrier.

So carriers containing ligands such as mannosyl, immunoglobulin, fibronectin and galactosyl are better phagocytosed by macrophages than carriers without such ligands<sup>16</sup>.

In the present study, gelatin was selected as the polymer for preparing nanoparticles because it is biocompatible and biodegradable<sup>13, 17</sup> and nanoparticles as a novel carrier system shows better accumulation in macrophage rich organs and better *in vivo* stability.

In the present investigation, gelatin nanoparticle bearing the antioxidant enzyme Catalase coupled with mannose is developed for controlled and site-specific delivery to macrophages to reduce the formation of ROS, which could be helpful in prevention of liver disorders due to ROS.

## MATERIALS AND METHODS:

**Materials:** Gelatin type A (derived from porcine skin, bloom 175, Sigma Chemical Co (St Louis, MO, USA), Bovine liver Catalase, and Mannose obtained from gift sample from Himedia (Mumbai). Glutaraldehyde, acetone, methanol, and acetonitrile were purchased from Spectrochem Pvt. Ltd. (Mumbai, India). All other reagents and solvents used were of analytical grade and procured from local suppliers.

### Methods:

**Preparation of Gelatin Nanoparticles:** The Gelatin nanoparticles were prepared by a two-step desolvation method<sup>23</sup> with suitable modifications. Briefly, 1.25 g gelatin was dissolved in 25 mL distilled water at 40° to 45°C until a clear solution was obtained, and then desolvated by the addition of 25 mL of acetone. Constant stirring (700 rpm) was maintained during the whole preparation procedure. The supernatant was discarded, and sediment was redissolved in 25 mL of distilled water. Then, 3mg/ml of drug solution was added to the above gelatin solution at 40°C and acidic pH 2.5 was maintained.

Then, 90 mL of acetone were added dropwise (3-5 mL/min) with stirring to ensure redesolvation of the polymer solution, which leads to the formation of nanoparticles. While stirring, 200 µL of 25 % aqueous glutaraldehyde v/v solution was added to effect crosslinking and stabilization of *in situ* nanoparticles. After stirring of 12 hours nanoparticles were purified by centrifugation (16000 g, 20 minutes) and redispersed in acetone/water (30/70). The purified nanoparticles are stored as dispersion in highly purified water at 2-8°C.

**Preparation of Mannose coupled Gelatin Nanoparticles:** Mannose coupled gelatin nanoparticles is prepared by the incubation method with few modifications<sup>19, 20</sup>. 1.0%, m/V mannose solution was prepared in hot water and mixed 1.0 mL of the preformed nanoparticle suspension with mannose solution and stirring this mixture overnight at room temperature. The prepared conjugate was separated using dialysis membrane of molecular weight cutoff of 100 kD. Distill water was used as dialyzing media with multiple washing and characterized by infrared spectroscopy.

## Nanoparticle characterization:

**Particle size:** The average particle size and polydispersity index of the coupled and uncoupled NPs were determined by Photon Correlation Spectroscopy using a Zetasizer (DTS Ver. 4.10, Malvern instruments, England). The sample was diluted 1: 9 v/v with deionized water. The particle size distributions are represented by the average size (diameter) and the variance (polydispersity) of the Gaussian distribution function in logarithmic axis mode (**Table 2**).

**Surface morphology (SEM):** Scanning electron microscope was used as a visualizing aid for surface morphology. The samples for SEM were prepared by lightly sprinkling the NPs powder on a double adhesive tape, which was stuck on an aluminum stub. The stubs were then coated with gold to a thickness of about 300 Å using a sputter coater. All samples were examined under a scanning electron microscope (LEO 435 VP, Eindhoven Netherlands) at an acceleration voltage of 30 kV, and photomicrographs were taken at 2800X (**fig. 1**).

**Particle Morphology (TEM):** Transmission electron microscope was used as a visualizing aid for particle morphology. The sample (10µL) was placed on the grids and allowed to stand at room temperature for 90 sec. Excess of fluid was removed by touching the edge with filter paper. All samples were examined under a Transmission Electron Microscope (Philips Morgagni 268, Eindhoven, Netherlands) at an acceleration voltage of 100 kV, and photomicrographs were taken at 1400X (**fig. 2**).

**Entrapment efficiency:** To calculate the amount of catalase incorporated into the nanoparticles, 1 ml dispersion was centrifuged at 16,000 g for 20 min and the sedimented particles were mixed with 1.0 ml of 0.2 M NaOH solution until a clear solution was observed (48 h) at room temperature. Catalase content was determined spectrophotometrically at 240 nm against a blank solution of unloaded gelatin nanoparticles<sup>18</sup>.

**Zeta potential ( $\zeta$  potential):** As the  $\zeta$  potential cannot be measured directly, it is calculated from the electrophoretic mobility of the analyte, according to the Debye-Hückel-theory and the Helmholtz-Smoluchowski-equation.

The zeta potential of the plain and coupled NPs was determined by Photon correlation spectroscopy using a Zetasizer (DTS Ver. 4.10, Malvern instruments, England). Measurements were performed in specific disposable cuvettes at concentration of 20µg/ml and recorded in Table 2.

**Drug release study:** The release of drug nanoparticles was carried out in PBS at 37°C in a shaking water bath. 20.2 mg of sedimented drug loaded gelatin nanoparticles were re-suspended in 5.0 ml of PBS. After different intervals of time, the particles were centrifuged and 3ml of supernatant was removed and determined spectrophotometrically at 240 nm against blank solution obtained by same process without drug. Percent cumulative drug release of optimized formulations is recorded in<sup>18</sup> **Fig. 4**.

**Estimation of Drug in Serum:** Blood was collected from cardiac puncture in a centrifuge tube containing heparin sodium (anticoagulant) and centrifuged at 5000 rpm for 10 minutes. Supernatant was collected and acetonitrile (1 mg/ml) was added to precipitate the proteins. The precipitated proteins were settled by centrifugation at 5000 rpm for 10 minutes and supernatant was collected. One ml of collected supernatant was filtered through 0.45-µm membrane filter and concentration was determined.

**Estimation of Drug in various Organs (Biodistribution Study):** Various isolated organs (liver, lungs, kidney and spleen) after drying using tissue paper were weighed and minced into small pieces. One gram of each organ was homogenized with 2.0 ml of PBS (pH 7.4). In the organs weighing less than one gram, whole organ was used. To tissue homogenate, 2ml of acetonitrile was added and kept for 30 minutes. The resultant suspension was centrifuged for 20 minutes at 5000 rpm and was filtered through 0.45-µm membrane filter. Tissue homogenate was analyzed for drug content in serum.

## ***In vivo* studies:**

**Selection of Animal:** *In-vivo* studies were performed on Balb/c mice, 6-8 weeks old, weighing between 20-25g and were divided into four groups. The animals were kept under standard conditions with free access to water and foods in accordance with Institutional Animal Ethics Committee.

**Experimental design and procedure:** To observe the antioxidant effect of formulation, hepatotoxicity is induced in mice by CCl<sub>4</sub> administration. CCl<sub>4</sub> dissolved in sesame oil (2% v/v) was administered to the peritoneal cavity of mice at a dose of 10 ml/kg body weight to induce acute liver failure. At predetermined periods after injection, blood was collected and the serum glutamicoxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activities were determined<sup>21</sup>.

To examine the antioxidant effect, formulations were injected intravenously into the tail vein of mice immediately after CCl<sub>4</sub> administration, at 6, 12, 24 hrs after CCl<sub>4</sub> administration, blood was collected and enzyme levels were determined. Serum was separated by centrifuge 15000 rpm, 15 min. A portion of liver tissue was removed, washed with ringer solution to remove debris and dried using tissue paper and some portion homogenized with TRIS- HCL buffer (pH.7.4) at 3000 rpm for 3 min. The homogenate was transferred to a centrifuge tube, shaken using a vortex mixer for 1 min, and centrifuged at 2500 rpm for 15 min.

After centrifugation the supernatant was transferred and evaporated to dryness. The residues were reconstituted with acetonitrile: sodium chloride (0.9% w/v) (85:15) and catalase was estimated<sup>21</sup>. For organ distribution studies, after performing the experiment, animal sacrificed, and organ were removed and washed with ringer solutions to remove the debris and dried with tissue paper and tissue were homogenized in same manner and estimate the concentration in different tissue at the different time interval. In this experiment, first group kept as a CCl<sub>4</sub> induced mice, second group for plain drug solution and third for Gelatin nanoparticle and fourth was mannose coupled gelatin nanoparticle.

**Experimental protocol:** The animals of the first group was CCl<sub>4</sub> induced mice, drug solution was given to animals of second group, while 3<sup>rd</sup> and 4<sup>th</sup> groups animals were administered drug loaded NPs and drug loaded mannose coupled gelatin nanoparticle (**table 1**).

**TABLE 1: EXPERIMENTAL PROTOCOL**

<b>Group I</b>	CCl <sub>4</sub> induced mice
<b>Group- II</b>	CCl <sub>4</sub> +Drug solution
<b>Group III</b>	CCl <sub>4</sub> +Gelatin NPs
<b>Group IV</b>	CCl <sub>4</sub> +Mannose coupled-NPs

**Protective effect of Nanoparticles formulations against CCl<sub>4</sub> induced hepatotoxicity:** After administration of CCl<sub>4</sub> to mice, serum GOT and GPT activity started to increase at 12 hrs reached a higher level at 24 hr (**Fig. 8**). Because the higher level of the serum SGOT and SGPT activity were observed at 24 hr after CCl<sub>4</sub> administration and it was good enough for comparison, so the time point of 24 hr was selected to evaluate the effects of NPs-formulations in the following experiments. The administration of mannose coupled gelatin nanoparticle just after CCl<sub>4</sub> administration effectively show significant reduction the serum GOT and GPT level, Compared with the plain drug solution, & gelatin nanoparticles (fig. 8).

**Statistical analysis:** Statistical analysis of data was performed using the analysis of variance (ANOVA).

## RESULTS AND DISCUSSION:

**Preparation and Characterization of Nanoparticles:** The gelatin nanoparticle prepared by the two-step desolvation method showed a high stability, much less aggregation and more homogeneous as compared to one step desolvation method<sup>22</sup>. After preparation of NPs, the mannose was attached at the surface of preformed NPs of gelatin through linkage between -CHO groups of mannose with -NH<sub>2</sub> groups of gelatin nanoparticles. The coupling of gelatin with NPs was confirmed by comparing IR spectra of gelatin and gelatin NPs-mannose conjugates.

In case of mannose coupled gelatin nanoparticle, N-H bending of secondary amines at 1580 cm<sup>-1</sup> and C=N stretch at 1470cm<sup>-1</sup> shows the formation of Schiff's base (RCH=N-R bond), confirming the formation of a linkage between mannose ligand and amine termination of the nanoparticles. These prepared gelatin nanoparticle and mannose coupled gelatin nanoparticle were characterized for shape and surface morphology, particle size, drug entrapment efficiency and Zeta potential.

The SEM and TEM photomicrograph exhibit that NPs are spherical in shape but the surface of mannose coupled gelatin nanoparticle is less smooth as compared to their gelatin nanoparticle (**fig. 1 & 2**). The average size of gelatin nanoparticle was found to be 235±1.2nm which was increased to 295±7.4nm which could be due to coupling of mannose molecules at the surface of NPs.

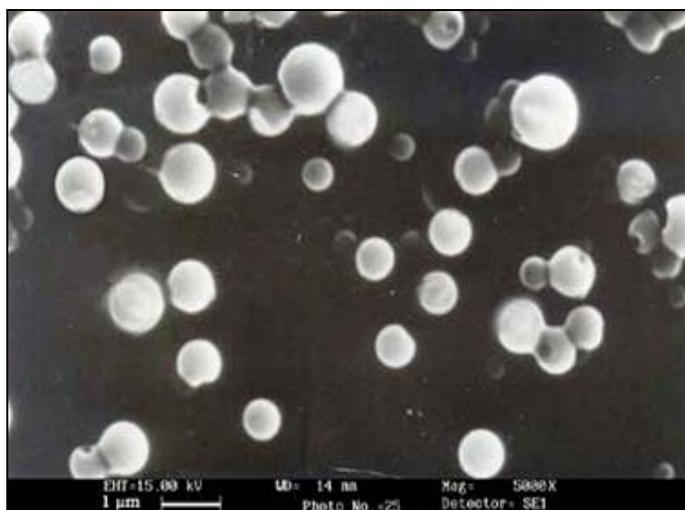
The drug entrapment efficiency of mannose coupled gelatin nanoparticle was found to decrease ( $61.81 \pm 1.4\%$ ) as compared to gelatin nanoparticle ( $68.56 \pm 2.4\%$ ). This could be due to leakage of drug during coupling process. The Zeta potential of

coupled mannose coupled gelatin nanoparticle was decreased on coupling of mannose to the surface of gelatin nanoparticle. The decrease in Zeta potential may be due to reaction of  $-\text{NH}_2$  group of gelatin with  $-\text{CHO}$  group of mannose (**Table 2**).

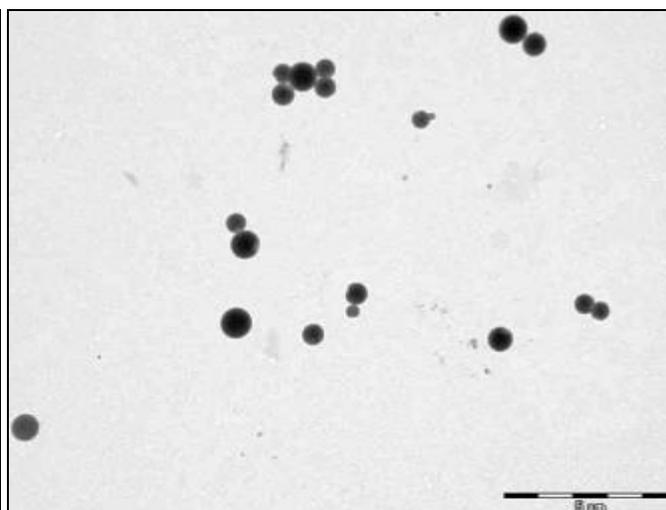
**TABLE 2: CHARACTERIZATION OF NPS FORMULATIONS**

Formulations Code	Particle size	Drug loading efficiency%	Zeta potential (mv)	Polydispersity index
Gelatin-NPs	$234 \pm 1.2 \text{ nm}$	$68.56 \pm 2.4\%$	$17.63 \pm 1.5$	$4.8 \pm 0.5$
Mannose coupled-NPs	$295 \pm 7.4 \text{ nm}$	$61.81 \pm 1.4\%$	$-16.77 \pm 1.1$	$5.7 \pm 0.8$

The values are mean  $\pm$  SD (n = 3).



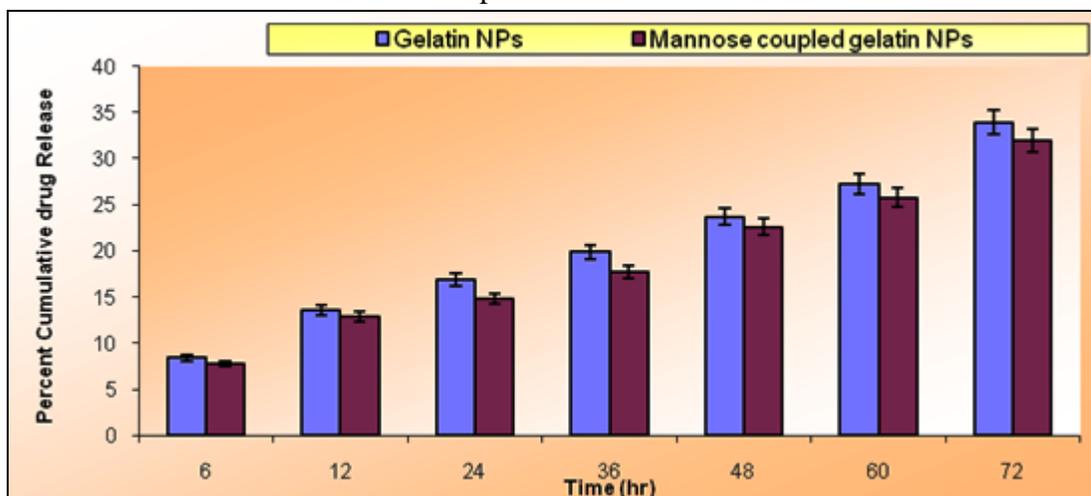
**FIG. 1: SEM PHOTOMICROGRAPH OF MANNOSE COUPLED GELATIN NPs**



**FIG. 2: TEM PHOTOMICROGRAPH OF MANNOSE COUPLED GELATIN NPs**

**In-vitro Drug release studies:** *In-vitro* drug release studies<sup>18</sup> indicate that drug release from optimized formulation was  $34.05 \pm 1.10\%$  after 72 hr in case of plain gelatin NPs while in case of mannose coupled

gelatin nanoparticle shows  $32.36 \pm 7.84$ . This could be due to presence of an addition barrier layer of mannose over the surface of NPs (**fig. 3**).



**FIG. 3: PERCENTAGE CUMULATIVE DRUG RELEASE FROM MANNOSE COUPLED GELATIN NANOPARTICLE & GELATIN NPs AT DIFFERENT TIME INTERVAL. (n=3;  $\pm$  SD)**

**Biodistribution studies in Serum and various organs:** Organ distribution pattern and serum concentration of catalase in case of gelatin nanoparticle, mannose coupled gelatin nanoparticle

and free catalase drug clearly establish the superiority of NPs formulations in reducing the concentration of free drug serum and in increasing the accumulation of catalase in the organs.

Biodistribution pattern of free drug, gelatin nanoparticle and mannose coupled gelatin nanoparticle formulations have been given graphically depicted in **Fig. 5-7**. For an easier understanding, the total percentage of administered dose present in serum as free drug at different time intervals was calculated. The calculated data has been graphically in **fig. 4**.

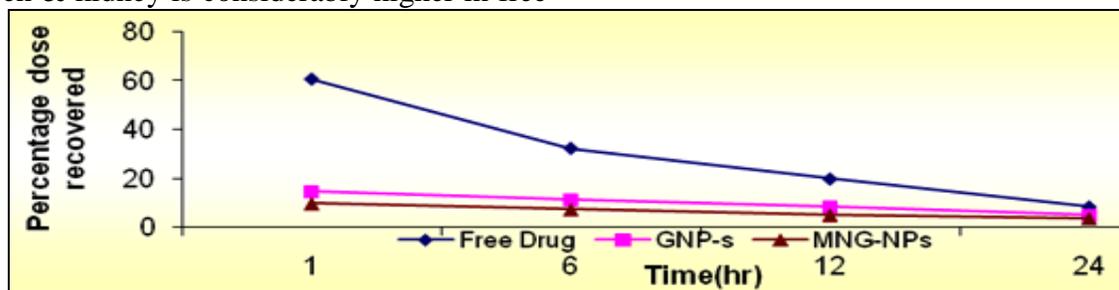
In case of free drug, maximum dose 60.6% was recovered in serum after 1 hour but in case of gelatin nanoparticle and mannose coupled gelatin nanoparticle dose recovered in serum was 14.7% and 9.8% after 1 hour, respectively. After 24 hours 8.4%, 5.2% and 3.8% drug was recovered respectively. These results clearly indicate the drastic reduction in serum concentration of drug entrapped in NPs, which is further reduced following the ligand coupling to the NPs.

The significant reduction in the serum concentration of free drug in NPs formulations can be accounted for the fact that the most of the drug present in blood is entrapped in NPs (Fig. 4). The estimation of amount of drug present in liver, kidney, lung, and spleen at different time interval after I.V. administration of free catalase revealed that maximum accumulation of the drug in these organs was achieved with in few hours. However, the maximum amount of drug accumulated in liver, lungs, spleen & kidney is considerably higher in free

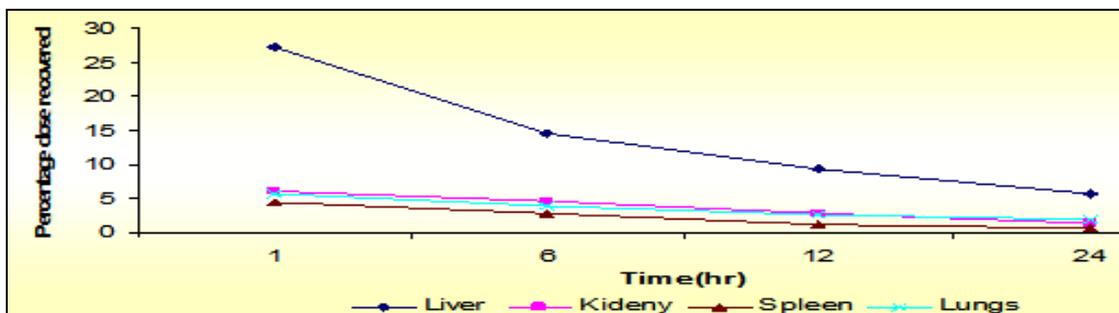
drug formulation than various coupled and uncoupled NPs formulations. As shown in fig. 5, free drug cleared quickly from the blood. In contrast, blood level in the case of gelatin nanoparticle and mannose coupled NPs remained high for a longer period, however; the blood level of mannose coupled NPs were lower than plain nanoparticles. This may be due to the relatively rapid uptake of mannose coupled NPs Ps by macrophage-rich organs in comparison to plain nanoparticles.

The estimation of amount of drug present in liver, kidney, spleen, lung, at different time intervals after intravenous administration of free drug revealed that maximum accumulation of the drug in these organs was achieved within 1–2 hours. The accumulation in different organs was  $12.11 \pm 0.2$  in lungs,  $22.45 \pm 0.4$  in liver,  $7.34 \pm 0.2$  in spleen, and  $6.89 \pm 0.3$  in kidney after 1 hour with the gelatin nanoparticle formulation and  $20.17 \pm 0.2$  in lungs,  $29.14 \pm 0.3$  in liver,  $16.61 \pm 0.1$  in spleen, and  $12.07 \pm 0.2$  in kidney after 1 hour in the case of the mannose coupled gelatin nanoparticle formulations (fig. 5-7).

The percent drug recovered from the liver following administration of mannose coupled gelatin nanoparticle at different time intervals was about 1.3-2.0 times higher than gelatin nanoparticle and about 0.1-5 times higher as compared to plain drug solution.



**FIG. 4: PERCENTAGE DOSE RECOVERED IN SERUM AFTER ADMINISTRATION OF VARIOUS FORMULATIONS AT DIFFERENT TIME INTERVALS. (n=3;  $\pm$  SD)**



**FIG. 5: PERCENTAGE DOSE RECOVERED IN VARIOUS ORGANS AFTER ADMINISTRATION OF FREE DRUG AT DIFFERENT TIME INTERVALS (N=3;  $\pm$  SD)**

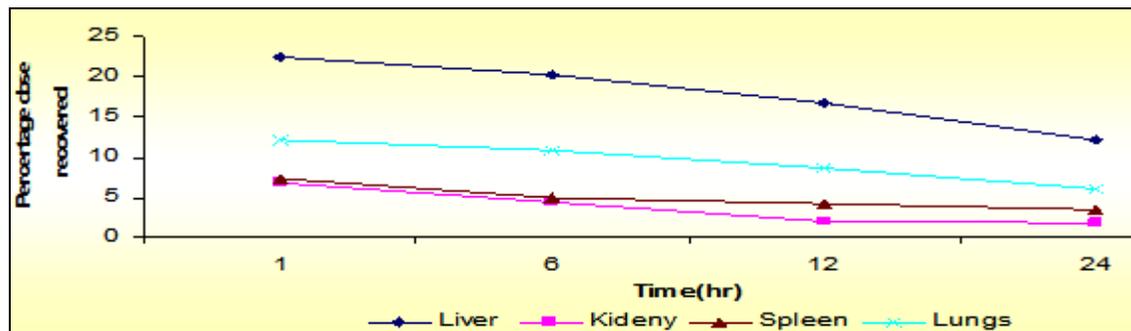


FIG. 6: PERCENTAGE DOSE RECOVERED IN VARIOUS ORGANS AFTER ADMINISTRATION OF GELATIN NANOPARTICLE DRUG AT DIFFERENT TIME INTERVALS (N=3;  $\pm$  SD)

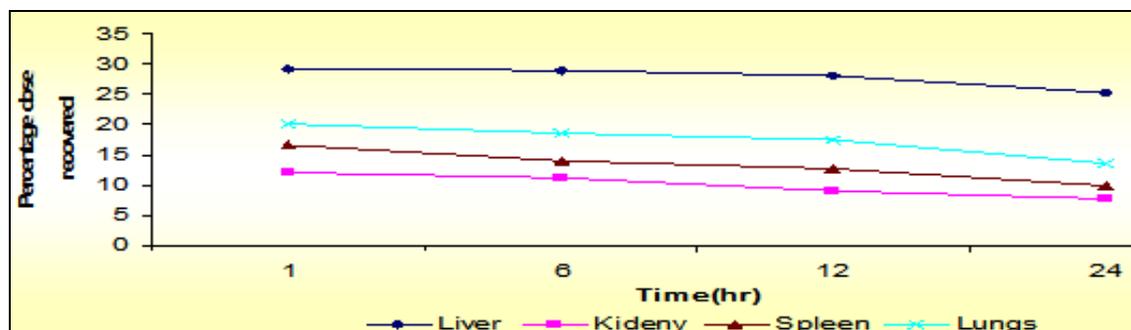


FIG. 7: PERCENTAGE DOSE RECOVERED IN VARIOUS ORGANS AFTER ADMINISTRATION OF MANNOSE COUPLED GELATIN NANOPARTICLE DRUG AT DIFFERENT TIME INTERVALS. (N=3;  $\pm$  SD)

**In vivo studies:** The selected gelatin nanoparticle and mannose coupled gelatin nanoparticle formulations were intravenously administered to BALB/c mice and compare of the drug plasma concentration profile and tissue distribution time profile and significant reduction of SGOT and SGPT enzyme level, with data obtained after the administration of plain drug solution was made. To examine the protective activity of NPs formulations the hepatic toxicity is induced in mice by  $\text{CCl}_4$  administration (fig. 8) which increases the level of SGPT and SGOT.

The serum glutamic oxalo-transaminase (SGOT) and serum pyruvic oxalo-transaminase (SGPT) are two enzymes, which are normally present inside the cells. When the cell is damaged these enzymes leak out and when a large number of cells are damaged their level in the blood will rise. It has been shown to rise clinically in myocardial infarction and in cases of liver damage.

Experimentally, it has been shown to rise when carbon tetrachloride injections are given to mice, Carbon tetrachloride causes damage to liver cells and necrosis of liver, in adequate doses. Using this very sensitive index it was decided to test the protective activity of mannose coupled gelatin nanoparticle

loaded catalase on the damage to liver cells caused by carbon tetrachloride.  $\text{CCl}_4$  is a classical hepatotoxin that causes acute, reversible liver failure characterized by centrilobular hydropic degeneration and necrosis. It is generally believed that this toxicity is due to lipid peroxidation caused by the highly reactive carbon trichloromethyl radical  $\text{CCl}_3$ , which is a metabolite of  $\text{CCl}_4$  produced by the P-450 enzyme system and it is characterized by elevated level of SGPT and SGOT enzyme level.

Thus, the protective activity and inhibition of free radical generation are important in terms of protecting the liver from  $\text{CCl}_4$  induced acute liver failure.

In the present study, mannose coupled gelatin nanoparticle containing CAT reduced the elevated serum GOT and GPT activities indicating its efficacy on preventing  $\text{CCl}_4$  induced acute liver failure in mice due to the significant detoxification of ROS. However, it has been reported that ROS including  $\text{H}_2\text{O}_2$  are generated after  $\text{CCl}_4$  administration.

Therefore, mannose coupled gelatin nanoparticle containing CAT can be effective in preventing  $\text{CCl}_4$ -induced liver failure by degrading  $\text{H}_2\text{O}_2$  generated in the process of  $\text{CCl}_4$  metabolism.

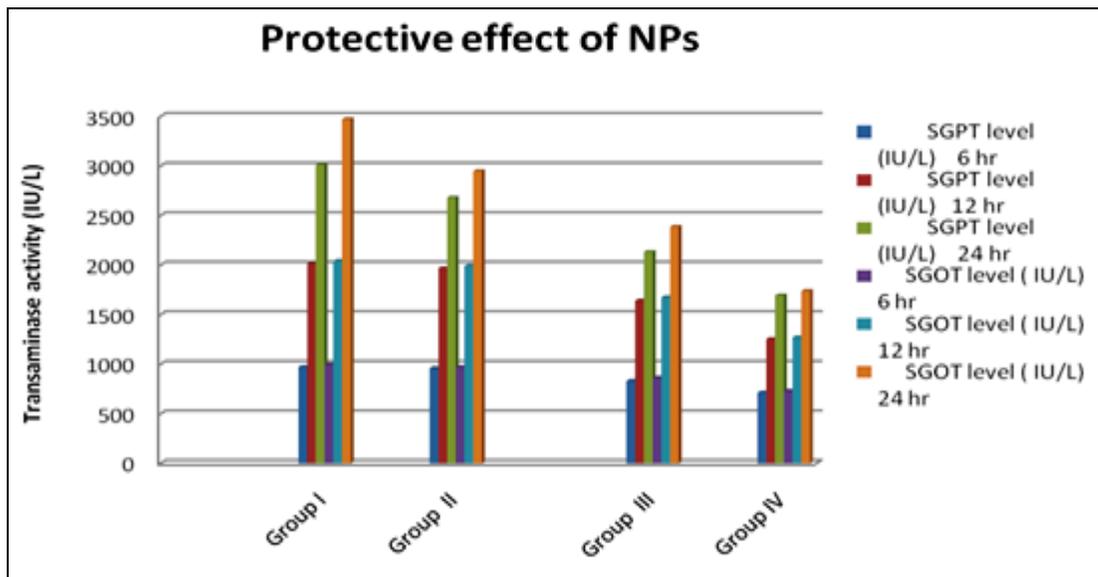


FIG. 8: PROTECTIVE EFFECT OF VARIOUS NPS FORMULATION IN SERUM TRANSAMINASE ACTIVITY (n=3;  $\pm$  SD)

**CONCLUSION:** Experimental studies revealed that greater accumulation of mannose coupled gelatin nanoparticles was observed as compared to gelatin nanoparticles in macrophages due to presence of mannose receptors in macrophages. Hence, it can be concluded that mannose coupling is as attractive and potential approach for targeting of CAT to macrophages and it has a significant potential to detoxify ROS. Therefore this approach could be further explored for the treatment of liver disorders arised due to ROS.

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