Chapter - VII
Engineered Nanoparticles in Diabetes Control
7.1 Introduction

Silymarin (Sm) is one of the oldest traditional herbal medicines used for centuries to combat different organ disorders. Sm is predominantly composed of four flavonolignan isomers; silybin, isosilybin, silychristine and silydianin. Among all, silybin (Sb) constitutes 34% of the mixture and is identified as the major biologically active component [166,167]. The most remarkable use of Sb is in treatment of acute mushroom (Amanita phalloides) poisoning where, Sb hepatoprotective effects have been demonstrated repeatedly in humans [176]. A large part of such effects are empirical and attributed to drug antioxidant properties. Sb however received much attention relatively recently because of other beneficial effects not directly related to its’ antioxidant properties. The flavonoid exerts anti-carcinogenic and anti-inflammatory effects and that are established up to the biomolecular levels [177,85]. Sb is recently proposed to be beneficial in type 2 diabetes patients and a number of articles relate decrease in both fasting and mean daily glucose, triglyceride and total cholesterol levels [178-180]. Additionally, aqueous extract of Silybum marianum exhibits potent hypoglycemic and antihyperglycemic activities in both streptozotocin and alloxan-induced diabetic rats [181,182]. Although the antidiabetic effects of Sb were observed at different levels, the underlying mechanism has not yet been fully explained. One early report relates Sb antidiabetic effects to inhibition of gluconeogenesis in the liver and decrease in glucose-6 phosphatase activity [183]. Another work classifies Sm as an agent for two way control against streptozotocin induced up regulation of cytochrome P 450 3A2 enzyme system and glutathione peroxidase down regulation [184]. Liver and pancreas centric effects of Sb in control of diabetes are generally accepted but appropriate delivery design remained a significant constraint both in systematic evaluation and applications. As like most flavonoids Sb suffers from significant solubility and biodistribution limitations. Delivery development for Sb is therefore one urgent need to reap benefit in diabetic control. Biochemical indications for Sb antidiabetic effects in addition could be helpful to provide a newer direction in management of one of the most rapidly progressing degenerative ailments in humankind.

PLGA is a safe US FDA approved biopolymer for applications in nanopharmaceuticals. However, the biopolymer undergoes a degree of hydrolysis during the passage in GIT. Cationic modifications however are known to enhance in appropriate pay load delivery. Moreover nanoparticle engineering with triblock pluronic biopolymers was earlier demonstrated to effect
higher mass payloading [185]. Conventional emulsion solvent evaporation technique experimented earlier (3.2.2) was not quite successful in Sb mass pay loading. High stress forces applied in homogenization and sonication were some of the reasons likely in loss of active ingredients in preparative stages. Besides, the stabilizer PVA used earlier is known to form interconnected network with the polymer at the interface which is difficult to be removed after emulsification [186,187]. A greater effect due to polymer and PVA stabilizer interaction was also observed in factorial design studies (4.3.1). Earlier analysis and observations incited us to exploit other techniques including nanoparticle engineering for loading an useful mass of the principle bioactive component Sb in PLGA nanoparticles. Acetone is one widely accepted solvent for PLGA nanoparticles due to its miscibility with water in all proportions [188,189]. Spontaneous diffusion of acetone in water is also known to create an interfacial turbulence resulting in formation of nanoscale particles. The principle was experimented for entrapment of Sb in PLGA. Another alteration instituted was application of a tri block polymer pluronic F-127 (or poloxamer 407) as a stabilizer in place of common agent PVA. Pluronic F-127 is recently approved by US FDA for application in drug delivery devices. In contrast to PVA, pluronics interact with both hydrophobic and hydrophilic domain and provide a brush-like coat which was considered to provide stability in nanoparticles both during preparation and in physiological environment [190]. The new nano-device designed with Sb as a pay load was further evaluated in streptozotocin induced diabetic model in rats.

7.2 Experimental Work

7.2.1 Preparation of chitosan modified silybin-PLGA nanoparticle (CSbnp)

In a typical experiment, 10 mg of silybin (Sb) and 50 mg of PLGA were dissolved together in 3 ml of acetone. The organic phase was then added to 30 ml of aqueous solution containing 1% w/v pluronic F-127. The addition was made by a syringe pump at a rate 15 µl/sec under magnetic stirring. Stirring was continued for an additional period of 12 h to evaporate off acetone. Nanoparticles were then recovered by ultracentrifugation at 30,000 rpm for 30 min at 4°C. The particles were then washed two times with HPLC water to remove unincorporated silybin, unbounded polymer and stabilizers.

Chitosan modified silybin nanoparticles (CSbnp) were prepared by polyelectrolyte deposition of chitosan. Briefly, silybin nanoparticles prepared as above were dispersed in HPLC grade water
and added dropwise into 0.1% w/v chitosan solution in 1% v/v aqueous acetic acid under magnetic stirring for 2 hour. CSbnp was recovered by ultracentrifugation, washed and redispersed. The empty nanoparticles were also prepared similarly but excluding only Sb.

7.2.2 Nanoparticle characterization

Particle size and zeta potential

The particle size, polydispersity index (PDI) and zeta potential of nanoparticles were measured in a Zetasizer Nano ZS (Malvern, UK) as detailed under section 3.2.3.

Atomic force microscopy

Atomic force micrographs of CSbnp suspensions were obtained in tapping mode using RTESP tip having resonance frequency 150-300 kHz at a scan speed of 1.2 Hz under Pico plus 5500 ILM (Agilent, USA) atomic force microscope. Images were captured and analysed using Picoview 1.10.4 software.

Transmission electron microscopy

For TEM measurements, 10 μl of CSbnp suspension in water were carefully placed on 300 mesh formvar-coated copper TEM grid (Ted Pella Inc., CA, USA) followed by staining with 2% w/v of uranyl acetate solution for 5 min. The excess solution on the grid was removed using a piece of fine filter paper and the samples were allowed to air dry for 10 h prior to imaging the particles under TEM (JEOL, Tokyo, Japan).

FTIR spectroscopy

FT-IR studies were carried out to detect each component interactions before and after nanoparticulation. Sb, chitosan, PLGA and the nanoparticles were pelletized individually with IR grade KBr, scanned over a range of 4000 to 400 cm^{-1} and the data stacked in Biorad KnowItAll software for analysis of overlap regions.

Silybin entrapment (%)

Estimation of Sb entrapment was carried out in a reverse phase HPLC system as discussed under section 3.2.3. Mobile phase used was 85% phosphoric acid: methanol : water (0.5:46:64 v/v/v) at
a flow rate of 1 ml/ min. Mass of total Sb in solution, before and after nanoparticulation in supernatant was determined in HPLC experiments for calculation of entrapment efficiencies.

Chitosan coating estimation

Quantitative estimation of cationic coating in CSbnp was performed using chitosan electrostatic interaction with alizarin red [191]. Stock solutions of chitosan of concentration of 1 mg/mL and of alizarin red S at a concentration of 1.5 mg/ml, were prepared. Different volumes of chitosan stock were taken in volumetric flasks and 0.8 ml of the dye solution was added to each flask. Volume of each flask was made upto 10 ml with sodium acetate buffer (pH 5). A standard graph from chitosan and alizarin reaction extinction recorded at 571 nm, was used to quantitate chitosan mass content both in primary stock solution and in final CSbnp preparation supernatants. The difference in mass was considered for estimation of chitosan coating. Experiments were run in triplicate in each case.

7.2.3 Release kinetics and modeling

For drug release studies, CSbnp equivalent to 9 mg of Sb payload, dispersed separately in 5 ml of phosphate buffer (100 mM, pH 7.4) was transferred into dialysis bags (MW cut off 12.4 KD) and was placed in glass vials containing 100 ml of phosphate buffer at 37°C in a shaker bath running at 70 rpm. At predetermined time intervals, 10 ml of phosphate buffer solution was removed for analysis and the release medium replaced with fresh buffer medium in order to maintain the sink conditions. The Sb mass released at definite time intervals was estimated in HPLC and were adjusted for the dilution factors. The release data was fitted to Korsemeyer-Peppas model and n and K values were calculated using sigma plot 6.0 software to understand the release mechanism.

7.2.4 In-vivo studies in streptozotocin (STZ) induced diabetic rat model

Animals

Forty male Wistar rats weighing 170-200 g were procured from Central Research Institute, (Kolkata, India). Animals were acclimatized under standard laboratory conditions of relative humidity 50 ± 10 %, temperature 22 ± 2°C, and 12/12 light dark cycle for 2 weeks prior to the start of the experiments. Access to water was ad libitum and standard pellet food (Hindustan Uniliver, India) supply was provided twice a day. All animal experiments were conducted as per
the approval of IAEC of University of Calcutta (Registration No. 506/01/a/CPCSEA, 2009-2010) vide its approval No 506/01/a/CPCSEA/CUTech03, dated 09/02/2010.

**Glucose tolerance test (GTT)**

Glucose tolerance test was carried out at the start of the experiment and at the end of the third week of experiment. This has helped to access the glucose homeostasis in normal conditions and finally after treatment. GTT measures the body’s ability to utilize glucose and is used to detect stages of pre-diabetic condition and to investigate postprandial glucose levels in a physiological way. Animals were fasted for a period of eight hours prior to analysis with water *ad libitum*. Each test animal was then challenged intraperitonealy with a freshly prepared aqueous D-glucose solution (2.0 g/kg). Blood glucose level was measured from the tail vein samples after 30, 60, 90 and 120 min of glucose injection and also initially at the 0th time. Individual animal data were grouped for ten animals in each treatment group and tabulated.

**Induction of diabetes**

Before the induction of diabetes, the animals were weighed and basal blood glucose level was measured. Diabetes was induced in overnight fasted groups by single intraperitoneal injection of streptozotocin (STZ) at a dose of 50 mg/kg body weight in freshly prepared ice-cold citrate buffer (0.1 M pH 4.0) [185]. These animals were fed with standard pellet food and a 5% glucose solution *ad libitum*, for 72 h. Afterwards, the glucose solution was replaced by water. The control rat received the vehicle alone. The diabetic state was assessed by measuring fasting glucose level of blood taken from the tail vein. Rats with a glucose level above 250mg/dl were considered as diabetic rats and used in further experiments.

**Animal treatment**

Rats were divided in four groups of 10 animals each in the following treatment schedule.

- Group 1 – Normal control received normal saline only 10ml/kg, b.w (C, nondiabetic)
- Group 2 – STZ induced diabetic rats used as diabetic control (D, diabetic)
- Group 3 – STZ induced diabetic rats treated with Sb 50mg/kg b.w for 28 days in an alternate day treatment schedule (SbT, diabetic and Sb treated)
Group 4 - STZ induced diabetic rats treated with CSbnp equivalent to 50mg/kg Sb payload for 28 days in an alternate day treatment schedule (CSbnpT, diabetic and CSbnp treated)

**Blood glucose estimation**

Fasting blood glucose concentration was monitored in 2 µl blood samples extracted from the tail of animal every week during the entire experimental period and at the end of treatment in the morning at 10 am using a portable glucometer (Dr. Morepen Gluco One Blood glucose monitoring system BG 03) with maximum measuring capacity of 600 mg/dl.

**Biochemical analysis**

At the end of treatment, rats were fasted overnight and the blood samples were received by cardiac puncture under light anaesthesia using diethyl ether. Samples were collected in marked vials added with or without anticoagulant for plasma and serum analysis and were stored at −20°C until further studies. The animals were finally euthanized at the end of the experiment by using CO₂ gas. The liver specimens were immediately removed and rinsed with chilled normal saline and cut into two parts. The first part following snap freeze in liquid nitrogen, was stored at −80°C for further molecular and biochemical analyses and the second half was kept in 10% formalin for histopathological examinations.

**Cholesterol, triglyceride and insulin in blood**

Serum insulin level was measured in blood using enzyme linked immunosorbent assay (ELISA) rat insulin kit (DRG diagnostics, Germany) [192]. Cholesterol and triglyceride levels in serum were estimated by using estimation kits (Span diagnostics limited, India) based on the methods of Wybenga *et al.* and McGowan *et al.* respectively [193-195].

**Estimation of total protein**

Total protein was estimated in serum and in liver tissue using bovine serum albumin (BSA) as a standard following the method of Lowry *et al.* [196].
Determination of the serum level of the hepatic marker enzymes
The serum level of aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were measured using commercially available standard kits (Span diagnostics limited, India) following the manufacturer’s instructions.

Estimation of oxidative stress markers and activities of antioxidant system

Determination of serum lipid peroxidation
Serum malondialdehyde (MDA) level as a measure of lipid peroxidation was assayed in the form of thio barbituric acid (TBA) reactive substance following the method of Yagi [197]. Briefly the reaction mixture containing 0.4 ml serum was diluted with 0.1ml distilled water. 2.5 ml of trichloroacetic acid (TCA, 1.22 M dissolved in 0.6 M HCl) was added in the serum sample and the mixture was left at room temperature for 15 min. 1.5 ml of 0.76% TBA containing 0.05 M NaOH was then added to the mixture and incubated in boiling water bath for 30 min. The reaction mixture was cooled and 4 ml of n-butanol was further added into it. The resultant chromophore was extracted from butanol phase. The generation of MDA was measured from the fluorescence emission intensity of the resultant chromogen 553nm by excitation at 515nm. The results were expressed in fluorescence units for comparison.

Estimation of SOD, catalase and GSH in liver tissue
Liver tissues were minced and homogenized (tissue homogenizer, TH 02, Omni International, Kennesaw, GA) in 10 mM potassium phosphate buffer containing 0.1mM EDTA, pH 7.4, at a proportion of 1:9 (w/v). The homogenate was centrifuged at 6000g for 10 min at 4 °C. The resultant supernatant was used for the determination of catalase, superoxide dismutase (SOD) and GSH activity.

SOD activity was measured according to the method of Marklund and Marklund [198]. In this test, the degree of inhibition of pyrogallol auto-oxidation by the supernatant of the tissue homogenate was measured. One unit of enzyme activity was defined as the amount of enzyme necessary for inhibiting the reaction by 50%. The enzyme activity was expressed as units per gram of tissue.
The catalase assay was carried out as described by Aebi 1984 [199]. Briefly, 50 μl of tissue homogenate was taken with 2950 μl of phosphate buffer in different test tubes (10 mM, pH 7.4). Hydrogen peroxide (H₂O₂, 80 μl, 10 mM) was further added to initiate the reaction. A blank was prepared with 2920 μl of the phosphate buffer and 80 μl of H₂O₂ without tissue homogenates. The decrease in optical density due to decomposition of H₂O₂ was measured at the end of 1 min using the blank at 240 nm. Enzyme activity was defined in terms of units of catalase required to decompose 1μM of H₂O₂ per minute at 25°C. The specific activity was expressed in terms of units/mg of tissue.

Tissue GSH was estimated following Ellman’s method with some modification [98]. Briefly, 200 μl of sodium EDTA solution (20mM) was added into 200 μl of tissue homogenate and was kept for 10 min at 4 °C. 400 μl of 5% TCA was further added and the mixture was left for 5 min at room temperature. After centrifugation, 500 μl of supernatant was collected and 500 μl of Tris buffer (200mM, pH 8.4) and 50 μl 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB, 100mM) were added. Optical density was monitored after 3 min at 412 nm against deionized water. A blank reaction run was also performed by mixing 500 μl tris buffer and 50 μl DTNB and the average in optical density at the beginning and at the end of the reaction time was recorded. The blank reading was subtracted from each sample readings and the results were expressed as μM/mg of tissue.

**Determination of the glycogen content in liver.**

The glycogen content was estimated according to the method by Murat and Serfaty [200]. Briefly, liver tissues were homogenized in ice-cold citrate buffer (0.1 M, pH 4.2), followed by centrifugation at 10,600g for 30 min at 40°C. The free glucose content in the supernatant was then measured [201]. Amyloglucosidase (2 mg, Sigma, USA) was added with the homogenate and was further incubated for 4 h at 37 °C. The total glucose content after incubation was then measured similarly. The glycogen content in the liver was calculated as the difference between total and free glucose.
Glycohemoglobin (HbA1c) whole blood

HbA1c in blood was estimated by the ion-exchange resin method [202, 203], using commercially available kit (Glycohemoglobin Unitest Set, EAGLE Diagnostics, USA). Whole blood was mixed with lysing reagent and the hemolysate was prepared and mixed with cation exchange resin. Hemoglobins (Hb) were retained by the resin, and HbA1c (fast fraction) was eluted. The percentage of HbA1c was determined by measuring the ratio of absorbance of the HbA1c fraction and the total hemoglobin fraction in the hemolysate at 415 nm, and results were compared with that for a standard HbA1c preparation throughout the test.

Serum fructosamine

Serum fructosamine (Amadori product) was determined by nitroblue tetrazolium (NBT) reduction assay according to the method of Johnson et al, 1982 [204]. Briefly, 1mL of NBT reagent (0.5mM NBT in 0.2M sodium carbonate buffer pH 10.4) was added to the serum part and the mixture was incubated at 37 °c for 1 h. The absorbance was measured at 530 nm against a reagent blank. The concentration of fructosamine was calculated compared to 1-deoxy-1-morpholino-fructose (1-DMF) as the standard [205].

Advanced glycated end product (AGE):

RBC hemolysates were prepared after washing with normal saline (0.9% NaCl) followed by hypotonic lysis in deionized water. Hb was isolated and purified by sephadex G-100 column chromatography, pre-equilibrated with 50mM potassium phosphate buffer, pH 7.4. The concentration of Hb was measured from Soret absorbance with the extinction coefficient as 125mM⁻¹cm⁻¹ at 415 nm and the results were calculated on monomer basis [206].

A separate set of experiment was run to measure AGE under identical conditions with the diabetic control animals six weeks after induction of diabetes. AGEs in Hb were estimated spectrofluorimetrically from the fluorescence emission at 440 nm on exciting Hb samples at 370 nm [207].

Histopathological analysis

Liver and pancreas tissues were fixed in 10% v/v formalin and dehydrated in a series of ethanol solutions (70, 80, 90,100% v/v). The tissue samples were processed by using routine paraffin
block techniques in paraffin wax. The samples were then sectioned (≈ 5 μM) and were stained with hematoxylin-eosin and mounted with neutral DPX medium. Photograph of stained sections were captured with a camera attached to a light microscope (B1 series, Motic, Xiamen, China).

7.2.5 Statistical analysis
Data were expressed as the mean ± standard deviation (standard of mean). Biological analysis data were gathered in triplicates and were assessed following student’s t test. Statistical significance was recorded when p<0.01.

7.3 Results and Discussion
7.3.1 Nanoparticle characterization
Nanoparticulation of insoluble compounds is one strategy to improve upon water solubility for enhanced bioactivity and ease in specific administration [208]. PLGA nanocarriers with Sb payload were prepared by solvent diffusion of acetone in water environment. Some advantages of this technique were mild preparative conditions, avoidance of high stress force and apparent ease in scale up. Chitosan association on nanoparticle surface accounted for a sustained release and drug permeability enhancement. Average hydrodynamic diameter of the prepared CSbnp was recorded in DLS as 229.7 nm with the PDI was 0.124. This indicated uniformity in size distribution at preparation system. Presence of molecular layer of polycationic chitosan on PLGA was evident from positive zeta potential value of +21 mV. Silybin entrapment percentage in CSbnp was determined in reverse phase HPLC and was recorded as 92.11%. AFM analysis of CSbnp revealed smooth surface topography for particles with mostly spherical geometry (Figure 28). Some particle shape however was not well defined likely due to coalescence in the scale of observation. Transmission electron microscopy showed the nanoparticles in near spherical shape having the mean particle diameter of 184.6 nm. Interestingly, no aggregation or adhesion was observed under dry conditions in TEM. The diameter of the particles observed in TEM was relatively smaller than the hydrodynamic diameters observed in the DLS method. Similar size difference was reported by other researchers and was attributed to particle drying under TEM observations [64].
In FT-IR the characteristics the benzopyran ring vibrations for Sb was recorded at 1084 cm$^{-1}$ alongside the flavonolignan ketone response at 1634 cm$^{-1}$. The C-H deformation was at 821 cm$^{-1}$ and the aromatic ring stretching vibrations were at 1508 cm$^{-1}$ (Figure 29). In case of PLGA, the ester -CO response was distinct at 1757 cm$^{-1}$ while the biopolymer C-H stretching was recorded at 2997 cm$^{-1}$. Chitosan when scanned in FTIR responded, at 1656 cm$^{-1}$ and 1591 cm$^{-1}$ due to amide I and amide II vibrations. In CSbnp, a strong shift due to electrostatic interaction of amide I appeared to a lower wave number at 1624 cm$^{-1}$, and a feeble response for chitosan -NH was clear. Similar observations of amide I shift and loss of amide II response due to strong electrostatic interaction between biopolymeric -COO$^-$ and chitosan NH$_3^+$ groups were also evidenced in earlier findings [209]. Non-covalent conjugations were clear in CSbnp and the characteristics Sb functional group responses were observed but with reduced intensity due to polymer nanoparticulation.
Chitosan mass coating estimation

Chitosan mass coating was analyzed on the basis of free chitosan left in the supernatant initially and after particle separation. A standard curve $y = 0.0037x + 0.1649$, $R^2 = 0.9863$, originally developed from the concentration ($x$) vs. absorbance ($y$) due to chitosan alizarin reaction in acidic pH environment (pH 5) was used to quantify the chitosan mass. The percentage chitosan coating efficiency of CSbnp calculated on weight basis was 77.81 ± 3.23.

7.3.2 In vitro release

Time-dependent cumulative Sb percentage release from CSbnp was studied up to 720 h (Figure 30) and almost 85% of the initial Sb mass load was accountable during the study period. Release pattern was biphasic, initial fast release lasted up to 8 h and that was followed by a sustained and....
steady release phase. Chitosan forms an entangled network layer on the particle surface which restricts the entry of water as well as prevents diffusion of drug molecules from nanoparticles surface to the surrounding medium that reducing the initial burst [210]. Besides, the solubility of chitosan is pH dependent and at a of pH 7.4 it is practically water insoluble, which further reduced the rate of diffusion water in CSbnp’s. This initial fast release phase was followed by a sustained and steady release phase. Sb release from CSbnp was delayed, releasing 31% of Sb mass load by 120 h. Drug release from particulate delivery devices is generally associated with intersects of multiple phenomenon including drug diffusion, polymer swelling, polymer erosion and degradation. The Peppas release exponent ‘n’ value of 0.45 for CSbnp indicated a potential overlapping of multiple incidents [211], including drug diffusion and polymer swelling.

Figure 30. *In-vitro* dissolution studies for CSbnp.

7.3.3 CSbnp in streptozotocin (STZ) induced diabetic rat

Effect of silybin and CSbnp on streptozotocin induced hyperglycemia and intraperitoneal glucose tolerance test (IPGTT)

Fasting blood glucose was monitored in different groups of rats at different time intervals and was presented in Figure 31. Blood glucose levels of untreated diabetic rats (D) were significantly higher compared to those of control group (C). One week treatment of diabetic rats with Sb (SbT) resulted in reduction of blood glucose levels but failed to normalize even after 4 weeks.
treatment. Treatment with CSbnp (CSbnpT) provided a significant reduction of blood glucose level within one week. Four weeks treatment with CSbnp reduced blood glucose value to near normal levels.

![Blood glucose levels over time](image)

**Figure 31.** Weekly blood glucose concentrations in different groups during the 4 weeks experimental period. C- control group, D - diabetic group, SbT - Sb treated group, CSbnpT - CSbnp treated group. Results are expressed as mean± standard deviation. "P < 0.01 significant difference compared with D group. #P > 0.5 no significant difference compared with D group.

IPGTT was performed for observations after three weeks of treatment with Sb or CSbnp. Diabetic rats exhibited glucose intolerant behaviour in comparison with control (Figure 32). Blood glucose level of diabetic rat when treated with CSbnp did return to near normal levels in 120 min after glucose injection. No significant difference was observed between glucose tolerance curves of CSbnpT and the control group at the end of 3rd week of treatment. Sb treated group however also exhibited reversal of glucose level but not upto the normal values.
Figure 32. Intraperitoneal glucose tolerance curve for different groups of animals. C - control group, D - diabetic group, SbT - Sb treated group, CSbnpT - CSbnp treated group. Results expressed as mean± standard deviation. * P < 0.01 significant difference compared with D group.

Effect of Sb and CSbnp on body weight
After four weeks of treatment, body weight of the rats in the normal control group was significantly increased when compared to initial body weight (P < 0.01). However, the body weight of the rats in the diabetic group was significantly decreased when compared to the initial body weight (P < 0.01). Diabetic rats treated with Sb did not show any significant changes when compared to the initial body weight (P < 0.05). However, the diabetic rats treated with CSbnp effect an increase when compared to the initial body weight (P < 0.01; Figure 33).
Effect of Sb and CSbnp on serum insulin, cholesterol and triglyceride levels in diabetic rats

Induction of diabetes resulted in significant reduction in serum insulin levels compared to that in normal rats (C). The levels improved slightly upon treatment with Sb treatment but improvements were higher when the group was treated with CSbnp (P < 0.01). The level of serum cholesterol and triglyceride were significantly decreased at about 47% and 45% respectively in diabetic rat treated with CSbnp nanoparticles as compared to diabetic group indicating a recovery (Table 15).

**Table 15.** Effect of Sb and CSbnp on serum insulin, cholesterol and triglyceride levels in diabetic rats.

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Insulin (µg/lit)</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (C)</td>
<td>0.60±0.06</td>
<td>77.6±6.54</td>
<td>55±8.02</td>
</tr>
<tr>
<td>Diabetic (D)</td>
<td>0.17±0.01</td>
<td>152.6±9.73</td>
<td>167.67±14.13</td>
</tr>
<tr>
<td>Sb treated (SbT)</td>
<td>0.29±0.06*</td>
<td>108.2±10.12*</td>
<td>127.5±20.17*</td>
</tr>
<tr>
<td>CSbnp treated (CSbnpT)</td>
<td>0.57±0.11*</td>
<td>80±6.13*</td>
<td>92.67±10.09*</td>
</tr>
</tbody>
</table>

Results are expressed as mean± standard deviation. *P < 0.01 significant difference compared with D group.
Liver marker enzyme analysis

Liver enzymes like alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) are responsible for proper functioning of the liver. Hyperglycemia induced hepatocellular damage may lead to excessive leakage of these enzyme into blood stream. STZ induced hyperglycemia established liver damage as evidenced by the elevated level of serum ALT, AST and ALP enzymes. Diabetic animal treated with Sb and CSbnp however showed differential activity on reduction of liver marker enzymes (Table 16).

Table 16. Effect of Sb and CSbnp over liver metabolic enzymes.

<table>
<thead>
<tr>
<th>Animal group</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>ALP (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (C)</td>
<td>94±9.11</td>
<td>47.33±3.24</td>
<td>82.33±6.38</td>
</tr>
<tr>
<td>Diabetic (D)</td>
<td>168±11.57</td>
<td>90.33±7.18</td>
<td>213.66±17.86</td>
</tr>
<tr>
<td>Sb treated (SbT)</td>
<td>132.66±12.87*</td>
<td>72.16±6.48*</td>
<td>188.33±13.54*</td>
</tr>
<tr>
<td>CSbnp treated (CSbnpT)</td>
<td>109.16±9.57*</td>
<td>58±6.65*</td>
<td>129.83±11.5*</td>
</tr>
</tbody>
</table>

Results expressed as mean± standard deviation. * P < 0.01 significant difference compared with D group. P < 0.05 no significant difference compared with D group.

Effect of Sb and CSbnp on oxidative stress markers and antioxidant system in diabetic rats

Serum MDA estimation

The oxidative stress in animals was measured by markers like secondary products of lipid peroxidation such as thiobarbituric acid reactive species (TBARS). The free radical measures like (CH₃⁺) have a very short half-life but the stress indicators also extend interesting observations on bioactive molecular mechanisms relatively specifically in diabetic conditions. Serum MDA level was measured from thiobarbituric acid reactive substance (TBARS) formation following the method of Yagi [197]. MDA level increased significantly in diabetic group. Protective effect of Sb and CSbnp were evidenced but in CSbnp treated animals the MDA levels were much closure to that of the control group (Figure 34).
Evaluation of antioxidant status

An imbalance between reactive oxygen species (ROS) generation and the reduced activity of antioxidant defences or both of these phenomena might lead to oxidative stress in diabetic condition. Oxidative stress negatively affects the activities of catalase, SOD and glutathione peroxidase in the liver tissues and also elevate the peroxidation levels [212]. Total antioxidant status was measured by the amount of enzymatic (SOD, catalase) and non enzymatic (GSH) marker.

SOD is an enzymatic antioxidant that catalyzes the conversion of the superoxide anion to hydrogen peroxide and molecular oxygen. The observed decrease in SOD activity in diabetic control rats could result from inactivation by \( \text{H}_2\text{O}_2 \) or by glycosylation of the enzyme, which have been reported to appear in diabetes [213]. Catalase is a haem containing ubiquitous enzyme that catalyzes the reduction of hydrogen peroxides and protects the tissues against reactive hydroxyl radicals. Decreased activity of this enzyme in STZ-induced diabetic rats could be due to inactivation by superoxide radical and glycation of the enzyme [214]. Our study also revealed
that the cellular levels of SOD and catalase were significantly turned down in the diabetic group rat as compared to that in control (Table 17).

Glutathione (GSH) is a direct scavenger of free radicals as well as a co substrate for peroxide detoxification which protects the cellular system against the toxic effects of lipid peroxidation and also it has a versatile role in antioxidant defense. In diabetic control groups, the decreased GSH may be due to reduction in GSH synthesis or degradation of GSH by oxidative stress in diabetic animal [215]. Restorative effect of Sb and CSbnp were indicated by the improved antioxidant status. However CSbnp treated animals demonstrated much closer SOD, catalase and GSH levels to that of the control group (Table 17).

**Table 17. Effect of Sb and CSbnp over antioxidant status of liver.**

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>SOD (U/gm of tissue)</th>
<th>Catalase (mU/mg of tissue)</th>
<th>GSH (µM/mg of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (C)</td>
<td>7.64±0.98</td>
<td>146.5±8.90</td>
<td>4.02±0.31</td>
</tr>
<tr>
<td>Diabetic (D)</td>
<td>3.42±0.64</td>
<td>88.5±8.76</td>
<td>2.54±0.21</td>
</tr>
<tr>
<td>Sb treated (SbT)</td>
<td>5.64±0.78*</td>
<td>113.83±11.34*</td>
<td>2.93±0.12*</td>
</tr>
<tr>
<td>CSbnp treated (CSbnpT)</td>
<td>6.84±0.32*</td>
<td>134.16±8.74*</td>
<td>3.54±0.16*</td>
</tr>
</tbody>
</table>

Results expressed as mean± standard deviation. * P < 0.01 significant difference compared with D group.

**Estimation of glycogen content**

The liver glycogen concentrations were significantly increased in both Sb and CSbnp treated groups compared to that in streptozotocin induced diabetic group. Maximum rise in liver glycogen content was however observed in CSbnp treated group that could be reasoned due to nanoparticle size assisted transport enhancement of Sb in liver (Figure 35).
Figure 35. Effect of Sb and CSbnp on liver glycogen content. Results expressed as mean± standard deviation. * P < 0.01 significant difference compared with D group.

**Estimation of HbA1c and serum fructosamine**

Fructosamine is a ketoamine formed when the carbonyl group of glucose reacts with an amino group of proteins, forming in glycated serum proteins [216]. The Amadori product or fructosamine is the first stable product of protein modification by glucose and its levels in serum increases in diabetes [217]. Fructosamine can undergo oxidative cleavage resulting in the formation of advanced glycation end product (AGE). A significant rise in serum fructosamine level was observed in diabetic group in comparison to that in normal control. Sb treatment reversed the increased fructosamine levels to a lower value while CSbnp resulted in near normalization (Table 18).

The HbA1c level is an important indicator in diabetic condition. The average HbA1c level was found to be significantly higher in D group of rats than that in control C group. Diabetic rats treated with CSbnp for three weeks showed a considerable reduction of glycation level of haemoglobin compared to Sb treatment for the same period indicating a recovery under diabetic conditions (Table 18).
Table 18. Effect of Sb and CSbnp on glycohaemoglobin (HbA1c) and fructosamine levels in diabetic rats.

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Serum fructosamine (μmol/μg of protein)</th>
<th>% of HbA1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (C)</td>
<td>0.59±0.12</td>
<td>1.79±0.37</td>
</tr>
<tr>
<td>Diabetic (D)</td>
<td>2.72±0.64</td>
<td>5.88±0.58</td>
</tr>
<tr>
<td>Sb treated (SbT)</td>
<td>1.12±0.45*</td>
<td>4.33±0.12*</td>
</tr>
<tr>
<td>CSbnp treated (CSbnpT)</td>
<td>0.56±0.03*</td>
<td>2.24±0.24*</td>
</tr>
</tbody>
</table>

Results are expressed as mean± standard deviation. *P < 0.01 significant difference compared with D group.

**Advanced glycated end product (AGE) measurement**

AGEs in Hb from different groups were estimated spectrofluorimetrically from the fluorescence emission at 440 nm after excitation of extracted Hb samples at 370 nm. Diabetic group showed significant presence of AGE in comparison to CSbnp treated group (Figure 36). Near normal response of AGEs in CSbnpT group was strong indicator for directed Sb response against diabetes.

![Figure 36. Effect of Sb and CSbnp on formation of advanced glycated end product (AGE).](image-url)
Histopathological study

**Figure 37.** Histopathological examination of tissue sections (A) Normal Pancreas, (B) Diabetic Pancreas, (C) Sb treated pancreas, (D) CSbnp treated pancreas (Magnification 100x).

Histopathological analysis of STZ-induced diabetic pancreas tissue slices exhibited shrinkage of islet cells and the growth of adipose tissue. Pancreatic sections of control animals showed normal islet histoarchitecture featuring circular shapes with healthy cell lining. CSbnp treatment produced marked recovery of structural integrity islets of langerhans along with minimal presence of fat tissues compared to Sb treated group (Figure 37).

**Figure 38.** Histopathological examination of (A) normal liver, (B) diabetic liver, (C) Sb treated liver (D) CSbnp treated liver (Magnification 100X).
Liver sections of non-diabetic control animals exhibited regular hepatic architecture, prominent centrilobular vein, sinusoidal spaces and prominent nucleus. Sections of the diabetic rat liver shows accumulation of fat droplets with distorted morphology of centrilobular vein, hepatocytes, and occurrence of sinusoidal dilatation. Treatment with engineered nanoparticles stimulated significant revival of hepatic cytoarchitecture with reduction of fat droplets as well as sinusoidal abnormality compared to Sb administered animals (Figure 38).

Streptozotocin is known to accumulate selectively in pancreatic beta cells via the low-affinity glucose transporter-2 (GLUT2) in the plasma membrane [218]. Selective toxicity of this agent causes DNA alkylation, protein glycosylation and depletion of cellular energy stores (ATP) ultimately leading to beta cell damages [219-221]. Insulin biosynthesis, glucose-induced insulin secretion and glucose metabolism get affected as a result of impaired beta cell functions [222,223]. Increased blood glucose levels in diabetes results in fuel up production of free radicals and increased oxidative stress by activating mitochondrial NADPH oxidase [224,225]. Oxidative stress induced a decrease in the activities of catalase, SOD and glutathione peroxidase in liver tissues with elevated levels of peroxidation [212]. Upshot of oxidative stress prevents insulin transport through endothelial wall and limits the delivery of hormone to the tissues [226]. Silymarin is reported to be associated with strong antioxidant properties, regenerative effect on beta cells, stabilizing effect on cell membrane and enhancement of membrane permeability to glucose [227-229]. Recent studies have also demonstrate that Sb could protect pancreas from cyclosporine A-induced toxicity and effect potent hypoglycemic effects in both type 1 and type 2 diabetes mellitus [223,230]. Sb also dose-dependently reduces the glucagon-induced stimulation of both gluconeogenesis and glycogenolysis and induces a potent decrease in glucose-6-phosphate hydrolysis [183].

The results of the present study showed that administration of Sb has an overall beneficial effect on streptozotocin induced diabetics in rats. Biopharmaceutic enhancement of Sb through nanoparticulation was marked by the rapid down regulation of blood glucose within two weeks of treatment. Bioactive Sb also lowered blood glucose level but failed to normalize the hyperglycemic condition in contrast to the animals treated with engineered nanoparticles. CSbnp treatment exhibited normoglycemic conditions after third weeks of treatment. This indicated that
a preparation like CSbnp could serve beneficially in diabetic conditions at least as an adjunct therapeutic. Nanoparticulation of Sb favored size assisted passive transport, improved solubilization of Sb and enhanced cellular uptake resulting in improved efficacy of CSbnp in diabetic animals [104]. Sustained release of Sb from liver depot could also be one reason for decreased gluconeogenesis in CSbnpT as was reflected in the increased liver glycogen content in treated animals. Besides CSbnp induced improved antioxidant defense has helped to regenerate pancreatic beta cell population which consequently effected in insulin blood levels. Additionally chitosan is reported to possess hypolipidemic and hypoglycemic effects both in vitro and in vivo [231]. Excessive hepatic gluconeogenesis and glucose production are important contributors to diabetic hyperglycemia. Chitosan molecularly can down regulate liver phosphoenolpyruvate carboxykinase (PEPCK) and phospho-p38 protein expressions but alternately enhanced the phosphorylation of liver AMPK and liver glycogen content thus may have a bearing in reducing liver gluconeogenesis in STZ-induced diabetic rats [201]. Therefore the combined antidiabetic response in engineered nanoformulation could be a significant contributor in improvements over glycemic conditions.

7.4 Inference
Silymarin was traditionally used against degenerative conditions. Silybin, the principle bio-active component that was proposed earlier as an alternative therapy in diabetes was successfully nanoparticulated following particle engineering techniques. The molecular loading was higher at 92.11 % and the release profile in case of new engineered nanoparticles CSbnp was sustained. CSbnp contribution in diabetes control was profound and a remarkable response was recorded in test animal recovery to near normal insulin levels and reduced glycated haemoglobin parameters. Liver concentric CSbnp could be one reason to strengthen overall antioxidant defence mechanism and aid in beta cell regeneration. This affected in increased insulin availability and a concomitant protection for liver glycogen stores. Such conditions are also likely due to Sb induced interfering in neoglucogenesis pathways. The designed Sb nanomedicines therefore provided newer directions in diabetes management and the nanoparticles are likely to aid further in diabetic control at least as an adjunct therapeutic.