5.1. Materials

HPLC solvents were procured from Merck, India. Working standards of sitagliptin phosphate (SP) and rosiglitazone (Internal standard, IS) were received as a gift sample (India). Anti-CD4 monoclonal antibodies (anti-CD4 mAbs) were received as a gift sample (India). Millipore water procured from Milli-Q RO system (Millipore, Bangalore, India). Poly (D, L-Lactide-co-Glycolide), pluronic F 127 were purchased from Sigma-Aldrich (Bangalore, India). Acetone, dimethyl sulfoxide (DMSO) of AR grade, streptozotocin (STZ) and dialysis bag (Cellophane membrane, M.wt cut off 10,000-12,000) was purchased from Hi-Media (India). Cystamine dihydrochloride, RPMI-1640 media, 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), phosphate buffer saline (PBS) were procured from Sigma-Aldrich (Bangalore, India). Tris (2-carboxyethyl)-phosphine hydrochloride (TCEP), m-maleimidobenzoyl-N-hydroxy-sulfosuccinimide ester (sulfo-MBS) were obtained from Thermo Scientific Pierce (India). Bayer Contour glucometer and Ts test strips were procured from local medical stores. Interferon-γ (IFN-γ) and interleukin-2 (IL-2) ELISA kits were obtained from Komabiotech (Seoul, Korea). Insulin and active GLP-1 ELISA kits were purchased from Millipore (India). All other chemicals were of AR grade obtained from Sigma-Aldrich (Bangalore, India).

5.2. Animals

Healthy adult BALB/c mice (20–30 g) were obtained from the central animal house facility of JSS College of Pharmacy, Udhamandalam, Tamil Nadu, India. The animals were kept in a well-ventilated room and animals had exposed to 12 h day and night cycle with a temperature 20 ± 3°C. The animals were housed in large spacious, hygienic polypropylene cages during the course of experimental period. The animals were fed with rat pellet feed (Hindustan Lever Ltd., Bangalore, India) and water ad libitum. All the experiments were performed after obtaining prior approval from Committee for the Purposes of Control and Supervision of Experimental Animals (CPCSEA) and Institutional Animal Ethical Committee (Proposal No. JSSCP/IAEC/PH.D/PH.BIOTECH/02/2012-13).
5.3. Preformulation studies

5.3.1. Development of analytical and bio-analytical method by HPLC

The HPLC method was developed to characterize and estimate the drug. The principle involved in the method is either adsorption or partition. The methods were developed using Shimadzu LC20AD HPLC system and the LC solutions software. Princeton SPHERE C_{18} column was used as stationary phase with ACN (Acetonitrile) and 0.5% TEA (Triethylamine) pH 6.8 as mobile phase at ratio of 50:50, and 23:77 v/v at a flow rate of 1.0 ml/min for analytical and bioanalytical methods, respectively. The pH of TEA was adjusted with orthophosphoric acid.

5.3.2 Curve of Linearity:

For development of the analytical curve, SP was dissolved separately in millipore water to get a concentration of 1 mg/ml solution. The further dilution was made with the same solution and the range of concentration considered for analytical curve was 5-50 μg/ml.

The bio analytical curve of sitagliptin was developed by spiking 0.5ml of sitagliptin into a mixture of 0.5ml of IS (Rosiglitazone), 0.5ml of plasma and 0.5ml of methanol (protein precipitating agent). Protein precipitation technique was adopted for the extraction of plasma samples. The range of concentration considered for bioanalytical curve was 5-500 ng/ml. The concentration of IS was maintained at 500 μg/ml.

5.3.3. Development of analytical method for anti-CD4 mAb by HPLC

The method was developed using Shimadzu LC20AD HPLC system and the LC solutions software. Princeton SPHERE C_{18} column was used as stationary phase with 20mM KH2PO4 (pH-6.8) : ACN (50:50, v/v) as mobile phase at a flow rate of 1.0 ml/min.

5.3.4. Curve of Linearity for anti-CD4 mAb

For development of the analytical curve, anti-CD4 mAb was dissolved separately in phosphate buffered saline to get a concentration of 1 mg/ml solution. The further dilution was made with the same solution and the range of concentration considered for analytical curve was 1-50 μg/ml.
5.3.5. Compatibility Studies by FT-IR:

The drug polymer interaction study was carried out by Fourier-transfer infrared (FT-IR) spectrum analysis. The Fourier-transfer infrared spectra's of dry samples maintained at isothermal stress conditions were recorded on Shimadzu FTIR. The KBr pellet method was employed as FTIR sampling technique. The samples - pure drug, polymer and the physical blend of drug and polymer stored at isothermal stress condition were mixed with KBr (IR grade) at ratio of 1:5 by weight. A thin layer pellets were prepared by subjecting samples to the hydraulic press at 10000-12000 Kg/cm² pressure. The analysis was carried in the frequency range between 4000-400 cm⁻¹.

5.3.6. Compatibility Studies by DSC:

The physical status of SP along with the combination of polymer stored at isothermal stress conditions was investigated by DSC, prior calibrated with indium as a standard with melting point at 156.63°C. The samples (pure drug, polymer and the physical blend) were accurately weighed (2-3 mg) and heated in closed aluminium crimped cells at rate of 20°C/min ranging from 30 – 300°C under purge of dry nitrogen at flow rate of 40 ml/min.

5.4. Formulation of sitagliptin loaded nanoparticles

Sitagliptin loaded polymeric nanoparticles (SP-NPs) were prepared by simple nanoprecipitation cum solvent evaporation method. In nano-precipitation method [99], phosphate buffer with pH 7.4 was used as external medium instead of aqueous phase. In brief, 50 mg of PLGA was accurately weighed and dissolved in 5 ml acetone. This organic solution was added slowly to 10 ml solution of Pluronic F 127 (1%) in phosphate buffer (pH 7.4) contains 10 mg of SP. The organic solvent was then allowed to evaporate for 4 h with continuous stirring on a magnetic stirrer (Remi). The NP suspension was then centrifuged and the sediment was freeze-dried. Nine batches of SP-NPs were formulated keeping the drug amount (X1) constant and varying polymer (X2), internal phase (X3) and surfactant concentrations (X4). The formula for nine batches was illustrated in table 1.
Table 1: Formula for SP-NPs batches

<table>
<thead>
<tr>
<th>Batches</th>
<th>X1 (mg)</th>
<th>X2 (mg)</th>
<th>X3 (ml)</th>
<th>X4 (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>A2</td>
<td>10</td>
<td>20</td>
<td>3</td>
<td>0.5</td>
</tr>
<tr>
<td>A3</td>
<td>10</td>
<td>50</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>B1</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>B2</td>
<td>10</td>
<td>20</td>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td>B3</td>
<td>10</td>
<td>50</td>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>C1</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>C2</td>
<td>10</td>
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<td>1.5</td>
</tr>
<tr>
<td>C3</td>
<td>10</td>
<td>50</td>
<td>5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

5.4.1 Characterization of SP-NPs

5.4.1.1. Process yield
The process yield of the formulated batches was calculated by gravimetric method. The suspensions were centrifuged at 7500 rpm for 30 min at room temperature and the sediments were lyophilized. The mean % process yield of all the batches with standard deviation (n=3) was determined from the below formula.

\[
\text{Weight of nanoparticles} \\
\% \text{ Process yield} = \frac{\text{Weight of nanoparticles}}{\text{Total solid weight}} \times 100
\]

5.4.1.2. Determination of Particle size
The size analysis of nanoparticles was performed by laser scattering technique using Malvern Nano-ZS. The nanoparticles were dispersed in an aqueous solution and taken in a sample dispersion unit and stirred during the process to minimize the inter particle interaction. The particle size analysis spectrum is a mean observation of three scans performed at 25°C. In addition to the mean particle size, the system reports a polydispersity index.
5.4.1.3. Zeta potential
The zeta potential of the formulations was measured using Malvern Nano-ZS. The analysis was performed twenty times after 6-8 folds dilution with water to result in optimum signal intensity at 25°C. The average value was recorded and used for further interpretation.

5.4.1.4. Determination of loading and entrapment efficiency
The content of SP in NPs was assayed by HPLC analytical technique. Briefly around 5 mg of particles of each batch was dissolved in 2 ml of ACN (HPLC grade) under vigorous vortexing. The solution was centrifuged and the supernatant was further diluted with mobile phase to ascertain the AUC of them fall in the linearity range. The analysis was performed in triplicate for each formulation batch. The loading and entrapment efficiency was calculated from below given formula and expressed as percentage.

\[
\% \text{ Entrapment efficiency} = \left( \frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}} \right) \times 100
\]

\[
\% \text{ Drug loading} = \left( \frac{\text{Total drug} - \text{Free drug}}{\text{Total amount of nanoparticles}} \right) \times 100
\]

5.4.1.5. DSC analysis
DSC analysis characterized the physical state of SP entrapped into the NPs. Each sample was sealed in standard aluminum pans with lids and purged with air at a flow rate of 40 ml/min. A temperature ramp speed was set at 20°C/min, and the heat flow was maintained in the range of 30–300°C under inert nitrogen atmosphere and the thermo grams of SP-NPs were recorded.
5.4.1.6. Surface morphology

The nanoparticles were imaged by a scanning electron microscope (SEM) at an accelerating voltage of 20 kV. To avoid the misconception of overlay of particles with agglomeration the particles were dispersed in water and few drops of which is fixed on to stub by a double-sided sticky carbon tape. The dispersion was air-dried and coated with gold layer for 60 sec before scanning for an image.

5.4.1.7. In vitro release studies

Two milliliters of nanoparticle suspension (corresponding to 2.0 mg of SP) was placed in a dialysis bag (cellophane membrane, molecular weight cut off 10,000–12,000, Hi-Media, India), which was tied and placed into 20 ml of phosphate buffer (0.1 M, pH 7.4), maintained at 37°C with continuous magnetic stirring. At selected time intervals, aliquots were withdrawn from the release medium and replaced with the same amount of phosphate buffer. The samples were assayed by HPLC. The data obtained from in vitro drug release were fitted into various kinetic models to understand the mechanism of drug release from the NPs.

5.4.1.8. Accelerated Stability studies

The studies were carried out for B3 formulation as per WHO guidelines for zone IV (hot and humid). The studies were carried at 25 ± 2°C at 60 ± 5% RH. The entrapment efficiency was evaluated as a function of the storage time on sampling interval of first, second and third month. On sampling interval of 6th month even the particle size and in vitro release studies were determined. The procedure for all the functions studied remains the same as that stated earlier.

5.5. Coupling of anti-CD4 monoclonal antibodies with SP-NPs

Coupling of anti-CD4 monoclonal antibodies with SP-NPs (anti-CD4 mAb-SP-NPs) was carried out by a two-step process involving the thiolation of free carboxyl groups on the NPs surface, followed by the covalent attachment of anti-CD4 monoclonal antibodies to thiolated NPs via a sulfo-MBS cross-linker.
5.5.1. Thiolation of SP-NPs

Thiol (–SH) group was covalently bound to the SP-NPs by a two-step carbodiimide reaction. Briefly, 100mg of SP-NPs were suspended in 5 ml water, and the reaction was initiated by consecutively adding 10 ml of a solution of EDAC (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide) and 5ml of a cystamine solution. The final suspension was completed with water to 25ml and stirred under mild conditions for 24 h at room temperature. Thereafter, EDAC and non-reacted cystamine were removed by two successive centrifugations (12,000 rpm for 30 min). The reduction of disulfide bonds was carried out in 20 ml of phosphate buffer (pH 4.7) by adding 1 ml of a solution of TCEP (Tris (2-carboxyethyl)-phosphine hydrochloride) (6 mg/ml). After 3 h of incubation, the thiolated nanoparticles were purified by two successive centrifugations to remove TCEP and freeze-dried. The concentration of the –SH functions on the surface of NPs was determined spectrophotometrically at \( \lambda = 412\text{nm} \) using Ellman’s reagent. The concentration of –SH functions on the surface of NPs was calculated by using following formula.

\[
\text{Thiol concentration} = \frac{\text{Absorbance of sample}}{\text{Molar extinction coefficient}} \times \text{Dilution factor}
\]

5.5.2. Covalent attachment of anti-CD4 mAbs to SP- NPs-SH

Two milligrams of purified anti-CD4 mAbs were activated in PBS (pH 7.4) with sulfo-MBS (m-maleimidobenzoyl-N-hydroxy-sulfosuccinimide ester) for 45min at room temperature. Non-reacted sulfo-MBS was removed by centrifugation. Five hundred microliters of activated anti-CD4 mAbs were reacted for 1h with gentle shaking at room temperature with 500\( \mu \)l of SP-NPs-SH. Unconjugated mAbs were removed by two cycles of centrifugation. Finally, anti-CD4 mAb-SP-NPs were stored in PBS at 4°C and used within a week after preparation. The amount of anti-CD4 mAb conjugated to SP-NPs-SH was determined indirectly by measuring uncoupled mAb in the supernatant after a centrifugation step by HPLC at a wavelength of 238nm.
5.6. Characterization of anti-CD4 mAb-SP-NPs

5.6.1. FTIR studies
The preliminary qualitative analysis for the coupling of anti-CD4 mAbs to NPs was determined by FTIR observations. FTIR spectrums were recorded by placing the samples in liquid holder.

5.6.2. Particle size & zeta potential
The size analysis of anti-CD4 mAb SP-NPs was performed by laser scattering technique using Malvern Nano-ZS. The particle size is a mean of three scans performed. In addition to the mean particle size, the system reports a polydispersity index. The zeta potential of the formulations was measured using Malvern Nano-ZS. The analysis was performed twenty times and the average value was recorded.

5.6.3. Surface morphology
Surface morphology of anti-CD4 mAb-SP-NPs was carried out by transmission electron microscope (TEM) to confirm the coupling of anti-CD4 mAbs on the surface of NPs. A drop of anti-CD4 mAb-SP-NPs suspension containing 0.01% of phosphotungstic acid was placed on a carbon film coated on a copper grid. The copper grid was fixed into sample holder and placed in vacuum chamber of the TEM and images were recorded at low vacuum.

5.6.4. Efficiency of conjugation
In order to verify the efficacy of anti-CD4 mAb conjugation with NPs-SH, an experiment was performed using non-thiolated NPs incubated with activated anti-CD4 mAb, which gets adsorbed on to the non-thiolated NPs and used as a control. The amount of anti-CD4 mAb conjugated to NPs-SH was determined indirectly by measuring uncoupled mAb in the supernatant after centrifugation step using HPLC.

5.6.5. Hemolysis Study
Erythrocytes were isolated from sheep blood and they were dispersed in normal physiological saline to obtain a suspension (2 wt%). To each 10 ml volume of SP-NPs (test), anti-CD4 mAb-SP-NPs (test), deionized water (positive control) and normal
physiological saline (negative control), 1 ml erythrocyte suspension (2% w/w) was added and incubated at 37°C for 30 min. Then centrifuged for 20 min (1500 rpm) to remove the erythrocytes or cellular debris. The absorbance values (A) of supernatants at 545 nm were determined for the presence of free hemoglobin. The hemolysis percentage of erythrocytes was calculated based on the formula shown below. For the studies of erythrocyte agglutination the same samples were placed on a glass slide, covered by a cover slip and analyzed by an inverted microscope.

\[
\% \text{ Hemolysis} = \frac{A_{\text{sample}} - A_{\text{NC}}}{A_{\text{PC}} - A_{\text{NC}}} \times 100
\]

Where \( A_{\text{sample}} \) is absorbance of sample, \( A_{\text{NC}} \) is absorbance of negative control and \( A_{\text{PC}} \) is absorbance of positive control.

5.6.6. In vitro cytotoxicity study
Cytotoxicity of formulations on the pancreatic islet was determined using a MTT reduction assay. Pancreatic islets were seeded onto 96 well plates at a cell concentration of 2×10^3 cells/well in 160 μl of RPMI-1640 medium containing 5% FCS and were pre-incubated overnight. After pre-incubation, the islets were incubated at 37°C for 30 min without or with 20 μl of SP-NPs and anti-CD4 mAb-SP-NPs (dissolved in cold normal saline). Then 20 μl MTT solution (5 mg/ml in PBS) were added to each well and the plate was further incubated for another 4 h at 37°C. Supernatants were then discarded and 200 μl of DMSO was added to the incubation mixtures and mixed thoroughly to dissolve the dark blue crystal formazan. The absorbance at 570nm (formation of formazan) and 655nm (reference) were recorded with a spectrophotometer.
5.7. In vivo studies

5.7.1. Induction of autoimmune diabetes

Forty mg/kg STZ dissolved in citrate buffer was intraperitoneally administered daily into 4 numbers of BALB/c mice for 5 consecutive days to develop STZ-induced autoimmune diabetes. Diabetic mice were then sacrificed as donors of diabetogenic splenocytes for the following transfer experiments 28 days after the last injection of STZ. Spleens of STZ-induced diabetic mice were removed, teased apart, and pressed through mesh with PBS to produce a single-cell suspension and suspended in complete RPMI-1640 medium (containing 10% fetal calf serum, 5 mmol glutamine, 0.5 mol/β-mercaptoethanol, and 400 U/mL sodium penicillin) at the concentration of $5 \times 10^5$/ml after washed twice using PBS. Then, $5 \times 10^5$ splenocytes (0.1 ml) were placed in a total volume of 0.2 ml RPMI-1640/FCS per well in a flat-bottom 96-well micro culture plate with 20 U/mL interleukin-2 (IL-2). A 7 days' incubation was carried out at 37°C and 5% CO₂, with fresh medium supplemented with cytokines every other day. After cultured, diabetogenic splenocytes were harvested through centrifugation and resuspended in PBS for adoptive transfer or relevant assays.

The purity of CD4⁺ T cells was assessed by fluorescence-activated cell sorting (FACS) assay using FITC-labeled anti-CD3 mAb and PE labeled anti-CD4 mAb. Totally, $4 \times 10^5$ cultured splenocytes in 5% FCS/PBS were incubated with FITC-labeled anti-CD3 or PE-labeled anti-CD4 mAbs for 30 minutes at 4°C, respectively. Then, the cells were washed twice in PBS and analyzed with flow cytometry using Cell Quest. The result was expressed as the percentage of cells conjugated with corresponding mAb in cells assayed. Cell suspension (0.2 mL) containing $1 \times 10^7$ cells was intraperitoneally injected into normal BALB/c mice recipients co-treated with low-dose of STZ (40mg/kg) twice 24 hours prior to the transfer (Fig 2). Blood glucose levels (BGLs) from tail vein were measured once in a week following cell transfer. Diabetes was defined when BGLs were > 200 mg/dl.
Fig 2: A schematic representation for induction of autoimmune diabetes in BALB/c mice. STZ induced diabetic mice were used as donors of diabetogenic splenocytes. Splenocytes were cultured and analyzed by FACS for purity of diabetogenic CD4\(^+\) T-cells. Finally adoptive transfer of diabetogenic splenocytes into normal BALB/c mice.

5.7.2. Pharmacokinetics and tissue distribution study

T cell induced autoimmune diabetic BALB/c mice were used for the pharmacokinetic and tissue distribution studies.

The experimental animals were divided into 3 groups each contains twelve.

- **Group I:** SP solution (20 mg/kg, i.p)
- **Group II:** SP-NPs (20 mg/kg, i.p)
- **Group III:** anti-CD4 mAb-SP-NPs (20 mg/kg, i.p)

SP was dissolved in phosphate buffered saline in order to make SP solution. The formulations were also suspended in phosphate buffered saline and were administered via i.p. route. Approximately 0.5 ml aliquot of blood samples were collected via heart puncture at time intervals of 0 hr (Pre dose), 0.5, 1, 2, 3, 4, 6, 8, 10, 12 and 24 hr of post dose. Pancreas’ were collected immediately after cervical dislocation at above described time points and weighed rapidly. Tissues were kept into normal saline solution to remove blood or content and blotted dry with tissue paper. For drug-content analysis, each pancreas was homogenized in pH 7.4 phosphate-buffered saline. Blood and tissue samples were centrifuged at 10,000 rpm for 10 min and supernatants were collected and stored at -70 ± 2°C until analysis.
The pharmacokinetic parameters were calculated by non-compartmental analysis of individual concentration-time data using PK software an Add-on program to Microsoft excel. The pharmacokinetic parameters such as maximum plasma concentration ($C_{\text{max}}$) and the time to reach $C_{\text{max}}$ ($T_{\text{max}}$) were obtained directly from the plasma concentration-time curve. Elimination rate constant ($K_e$) was obtained from the least-square fitted terminal log-linear portion of the plasma concentration-time profile, elimination half-life ($T_{1/2}$) was calculated as $0.693 / K_e$, area under the plasma concentration time curve from 0 to 24 h ($AUC_{0-24h}$) was calculated by the linear trapezoidal rule, and area under the curve from 0 h extrapolated to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-24h} + C_t / K_e$, where $C_t$ represent the observed plasma concentration at the last measurable sampling time. All values are expressed as means ± standard deviation except the $T_{\text{max}}$, which is expressed as the median.

5.7.3. Pharmacodynamics study
5.7.3.1. Treatment and monitoring of blood glucose levels
Animals were divided into five groups each group contains six. The five groups divided as follows: control, negative control, anti-CD4 mAb treated, SP-NPs treated and anti-CD4 mAb-SP-NPs treated group. Control and negative control group treated with phosphate buffered saline. A schematic representation of treatment is illustrated in the Fig 3. Followed by the treatment BGLs were monitored for nine weeks from the starting day of treatment. Reversal of diabetes was considered if BGLs were found to be < 200 mg/dL.

![Treatment Diagram](image)

**Fig 3.** A schematic representation of treatment followed by adoptive transfer
5.7.3.2. Oral glucose tolerance test
A subset of mice was selected at random and underwent an oral glucose tolerance test (OGTT) followed by the 4-week treatment. Mice were fasted for 12 h prior to OGTT. Glucose load was administered via gavage at 2 g/kg of body weight. BGLs were measured from tail bleeds using glucometer 0 min (before administration) and 60, and 120 min after the administration.

5.7.3.3. Insulin & GLP-1 levels during OGTT
Insulin and GLP-1 levels were measured during OGTT by ELISA method.

5.7.3.4. Cytokine analysis
The role of adoptively transferred CD4+ T cells on the proliferation of cytokines was determined using ELISA method. Mice were sacrificed followed by 4 weeks treatment, blood samples were collected and serum was separated from them. The concentrations of IL-2, interferon-y (IFN-y), in serum were determined.

5.7.3.5. Immunohistochemical analysis
Animals were sacrificed followed by 9 weeks of treatment and pancreas were isolated and sectioned at 3 mm thickness and 5-10 µm slices, using a Leica RM 2135 microtome. Slices were divided into two sets, one set was stained with hematoxylin-eosin (HE)) and the other set with anti-insulin antibody to know the pathological changes in pancreas. Anti-insulin antibody staining reveals the percentage of positive insulin area, which is directly proportional to the percentage of positive beta cell area.

5.8. Statistical analysis
The data are represented as the mean ± standard deviation (SD). Two-way analysis of variance (ANOVA) with Bonferroni’s multiple comparisons test was applied for BGLs followed by adoptive transfer of splenocytes, BGLs followed by treatment, OGTT and cytokine analysis. One-way ANOVA with Tukey’s multiple comparisons test was applied for pharmacokinetics, bio-distribution, insulin and GLP-1 levels during OGTT and for immunohistochemical analysis of pancreas. Unpaired t test was applied for
efficiency of anti-CD4 mAb conjugation with NPs. \( P < 0.0001 \) was considered statistically significant.