4.1 Materials

The following chemicals, equipments, animals and cell lines were used for the experimental procedure:

4.1.1 Chemicals

Materials	Suppliers
Hepatitis B Antigen	Gift sample from Serum Institute Pune, India
Poly lactic-Co-glycolic Acid (PLGA)	Gift sample by EVONIK industry, Mumbai, India
Poly vinyl alcohol (PVA)	Sigma Aldrich, Mumbai, India
Dichloromethane (DCM)	Thermo Fisher Scientific, Mumbai, India
Chloroform ($CHCl_3$)	SD Fine-Chem Ltd., Mumbai, India
Mannitol	Loba Chem Pvt. Ltd., Mumbai, India
Sodium hydroxide (NaOH)	Sigma Aldrich, Mumbai, India
Sodium Chloride (NaCl)	SD Fine-Chem Ltd., Mumbai, India
Potassium dehydrogen orthophosphate	SD Fine-Chem Ltd., Mumbai, India
Sodium hydrogen orthophosphate	SD Fine-Chem Ltd., Mumbai, India
Potassium chloride	SD Fine-Chem Ltd., Mumbai, India
Concanavalin A	Thermo Fisher Scientific, Mumbai, India
Methanol	SD Fine-Chem Ltd., Mumbai, India
Coomassie blue	Sigma Aldrich, Mumbai, India
Sodium azide (NaN_3)	P.C. Chem Mumbai, India
Span 80	SD Fine-Chem Ltd., Mumbai, India
Heparin	Biological E. Limited, Hyderabad, India

4.1.2 Equipments

Equipment	Manufacturer
Electronic balance	Shimadzu Ax200, Japan
Vortex mixer	Fisher Scientific, India
Ultraturrex	T25 IKA, India
Water bath sonicator	PCI Analytics Pvt. Ltd, Mumbai, India
High pressure homogenizer	IKA T25 digital Ultra-Turrax, Germany
Ultrasonic homogenizer	Dr. Heilscher, Germany
Magnetic stirrer	Eltek Electronic, Mumbai, India
Ultracentrifugation	Beckman Coulter India Pvt. Ltd., India
Cooling centrifuge	Rami, Mumbai, India
Micro centrifuge tubes	VOLEX Plasticwares, Italy
Lyophillizer	Decibel Dynamic Ltd., India
Particle size analyzer	Beckman Coulter, USA
pH meter	Eutech Instruments, Mumbai, India
Digital microscopy	Zeiss, AXIO, Imager A1, Germany
Dialysis membrane	Cambell Electronics, Mumbai, India
HPLC	Waters, USA
Scanning electron microscopy	SEM, Zeiss, Evo Research Ltd., Japan
Transmission electron microscopy	Tenai G^2 20 Twin, FEI Company, Netherland
Atomic force microscopy	NT-MDT, Moscow, Russia
Fluorescent microscope	Thermifischer Scientific, Japan
Flow cytometry	Coherent, Palo Alto, CA, USA
Syringe filter (0.22 µm and 0.45 µm)	Milipore Corporation, MA, USA
Rotary shaker	Khera Instruments Pvt. Ltd., Delhi, India
Micro pipettes	Thermo Scientific, USA
Eppendroff tubes	Eppendrof Corp., NY, USA

4.2 Methods

4.2.1 Preformulation study

4.2.1.1 Development of analytical method

Most of the biological analyte samples found in non-volatile or in aqueous matrix form. Sample of Hepatitis B surface antigen (HBsAg) is also available in aqueous form. Various methods based on different techniques are available for the detection of HBsAg. Assessment of HBsAg polypeptide can be performed by sucrose centrifugation, size exclusion chromatography, HPTLC and RP-HPLC (Reverse phase high performance liquid chromatography) method. RP-HPLC is extremely useful, easy and common method for detection of antigen having an aqueous mobile phase and hydrophobic stationary phase.

- **RP-HPLC analytical method**

The HPLC method is the most efficient technique for the assessment of antigen in formulation. RP-HPLC method was used for estimation and followed the ICH guideline for validation of HBsAg antigen.

- **Instrumentation and analytical conditions**

A reverse-phase high-performance liquid chromatography (RP-HPLC) system with photo diode array (PDA) detector was used for the in-vitro quantification and estimation of HBsAg. The HPLC system comprised of inline degasser, a Rheodyne 7725i manual injector, a 515 HPLC binary pump, C18 reverse phase column (250 mmX4.6 mm, 5 µm) protected with guard column. Methanol and phosphate buffer (pH 7.4) in the ratio of 50:50 (V/V) was used as mobile phase. Mobile phase was filtered through a 0.45 µm

membrane filter and pumped from the respective solvent reservoir to the column at a fixed flow rate 1.0 mL/min. The run time was set at 10 min and column temperature was maintained at 30 ºC. Antigen detection was performed at a wavelength of 280 nm (λ_{max}). The Peak area and retention time was determined by Empower Node 2054 software. Standard curves were plotted in the range of 1-25 µg/mL and validated for linearity, limit of detection and limit of quantification [Shrivastava and Jain, 2009].

- **Preparation of standard stock and working solutions**

1) Preparation of phosphate buffer saline, pH 7.4 (PBS): All below reagents are mixed and dissolved in 1000 mL of distilled water.

Reagents	Quantities
Disodium hydrogen phosphate	2.38 g
Potassium dihydrogen phosphate	0.19 g
Sodium chloride	8.0 g
Distil water	1000 mL

2) Sample solution preparation: Standard stock solution was prepared by dissolving accurately volume of 0.85 mL (in 1.74 mg/mL) of HBsAg in 5 mL of PBS, to give a 100 µg/mL concentration. Appropriate dilution of standard stock solution of antigen with an appropriate dilution was prepared in the range of 1-25 µg/mL. A standard calibration was constructed by plotting of mean peak area *vs.* antigen concentration of appropriate dilution. The HPLC method was validated for the quantification of drug in sample. Calibration curve was prepared and assayed in triplicate to evaluate linearity, limit of quantification (LOQ) and limit of detection.

- **Linearity and range**

The linearity range is expressed at lowest to highest levels of analyte and determined by measuring the absorbance of the sample analyte over the concentration. The calibration curve of Hepatitis B antigen were obtained with six working standard solution such as 1, 5, 10, 15, 20, 25 µg/mL. A calibration curve was constructed as linear plot of peak area verses antigen concentration. The experiments were performed in triplicates and the linearity was calculated with the help of linear regression analysis.

- **Limit of detection and quantitation**

The Limit of detection (LOD) is the lowest concentration of sample analyte that produces the assessable response whereas limit of quantitation (LOQ) is the lowest concentration/quantity of sample analyte at which it can not only be reliably detected. The formula has been used for the calculation of LOD and LOQ:

$$\text{LOD} = \frac{3.3}{\text{S}} \times \sigma$$

$$\text{LOQ} = \frac{10}{\text{S}} \times \sigma$$

Where, S is the slope of calibration curve and σ is the standard deviation of y-intercept of the regression equation.

4.2.2 Formulation of HBsAg loaded polymeric nanoparticles

Antigen-loaded nanoparticles were fabricated by double emulsion solvent evaporation method. Firstly, develop placebo nanoparticles (without drug) then prepared antigen loaded nanoparticles by double emulsion solvent evaporation method. The following reagents were required for preparation of nanoparticles:

- Antigen (Hepatitis B surface antigen)
- Organic Phase: DCM (Dichloromethane)
- Polymer: PLGA (Poly Lactic-co-Glycolic Acid)
- Emulsifier: Span 80
- Surfactant: PVA (Polyvinyl Alcohol), Span 80
- Cryoprotectant: Mannitol

Briefly, primary W/O emulsion was prepared by adding inner aqueous phase (pH 7.4 phosphate buffer saline containing antigen) to organic phase (dichloromethane containing PLGA) and sonicated for 2 min on (water bath sonicator, PCI Analytics Pvt. Ltd, Mumbai, Maharashtra) using span 80 as emulsifier agent. This prepared primary emulsion was again emulsified with aqueous solution of PVA and homogenized for 20 min at 10,000 rpm followed by sonication to obtain W/O/W emulsion. The resultant double emulsion was left for (5-6 hr) stirring on a magnetic stirrer at 300 or 600 rpm (room temperature) for evaporation of organic solvent. Nanoparticles were recovered by ultracentrifugation (Beckman Coulter India Pvt. Ltd, Andheri (East) Mumbai, Maharashtra) at 15,000 rpm for 20 min at 4 ºC. The supernatant was discarded to

remove free antigen and additional surfactant. Nanoparticles were washed twice with Milli-Q water and lyophilized [Pankhurst *et al.,* 2003; Nellore *et al.,* 1992; Singh *et al.,* 1997].

- **Optimization of process variables**

Optimization was done by selection of various dependent and independent variables. The effects of four different independent variable such as polymer concentration, percentage of PVA, Aq/Org. phase ratio and homogenizer speed on the dependent variables like particles size and entrapment efficiency were evaluated.

- **Experimental design model**

Experimental design model is the computer operated system program, using Design expert (7.0 aprobado) software. Design models were utilized for the optimization [Box and Wilson, 1951]. Another is response surface methodology (RSM) which consists of a set of statistical methods that can be used to develop, improve or optimize the process of production [Myers *et al.,* 2009]. Exact particle size and antigen entrapment efficiency of optimized formulation was found in two stages: in the first stage, parameters that showed significant effects on particle size and entrapment efficiency were taken and in the second stage, the optimum values of these parameters were analysed by using central composite design model (CCD).

Central composite design is a statistical experiment design, useful in three-level factorial experiment which can be utilized to select the most valuable parameters and their interactions in the experiments [Pawar *et al.,* 2010].

- **Screening study**

After selection of statistical and experimental design screening was done by identifying the most important parameters and their interactions [Pawar *et al.,* 2010]. In this dependent variables such as particle size (Y1) and entrapment efficiency (Y2) have been preferred as response variable for the optimization, while the polymer PLGA concentration (X1), PVA (X2) and ratio of Aq/Org phase ratio (X3) and homogenizer speed (X4) were preferred as the independent variables. Table 4.1 showed the list of independent, dependent variables and their corresponding levels. Total thirty experimental factorial formulae were generated which was the backbone of the design model. At the one centre point six replicates run were found according to the CCD.

Table 4.1: List of independent, dependent variables and their corresponding levels

		Levels of variables		
		Low	**Medium**	**High**
Independent variables	**Symbol**	**-1**	**0**	**+1**
Polymer (mg)	**X1**	**30**	**35**	**40**
PVA (%)	**X2**	**0.5**	**1.0**	**2.0**
Aq/Org ratio (mL)	**X3**	**2.0**	**2.5**	**3.0**
Homogenizer speed (rpm)	**X4**	**5000**	**10000**	**15000**
Dependent variables	**Symbol**	**Unit**	**Constraints**	
Particle size	**Y1**	**nm**	**Minimum**	
Entrapment efficiency	**Y2**	**%**	**Maximum**	

The four variables were studied at three levels like low, medium and high, which were denoted by conversion values of −1, 0 and +1, respectively. Table 4.2 showed combined effects of independent variables on particle size and entrapment efficiency.

When applying CCD model, two equations were obtained for the particles size and entrapment efficiency. Evaluation of these three-dimensional response surfaces graph was analysed by one-way analysis of variance (ANOVA) and calculation of the regression coefficients value. The multiple correlation coefficient (R^2) and adjusted R^2 were employed in composite design for evaluation of second-order polynomial equation as quality indicators. The model significance were selected as $P<0.05$ by Fisher's F-test. After solving the equation which was derived from the final quadratic model, optimal points were found and results were evaluated according to the graph. After resolving the equation responses based of independent variables were studied. Contour plot and three-dimensional surface plot were generated and trends in various responses with variables were studied. Desirability approaches were selected to obtain the optimum batches. Criteria are to be minimum particle size and maximum entrapment efficiency (%).

Table 4.2: Combined effect of independent variables on particle size and entrapment efficiency

Std	Run	Block	Factor 1 A: Polymer	Factor 2 B: PVA	Factor 3 C: Aq/Org. Ratio	Factor 4 D: H. Speed
4	1	Block 1	40.00	1.50	1.50	5000.00
7	2	Block 1	30.00	1.50	3.30	5000.00
9	3	Block 1	30.00	0.50	1.50	15000.00
27	4	Block 1	35.00	1.00	2.40	10000.00

20	5	Block 1	35.00	2.00	2.40	10000.00
16	6	Block 1	40.00	1.50	3.30	15000.00
12	7	Block 1	40.00	1.50	1.50	15000.00
29	8	Block 1	35.00	1.00	2.40	10000.00
3	9	Block 1	30.00	1.50	1.50	5000.00
19	10	Block 1	35.00	0.00	2.40	10000.00
30	11	Block 1	35.00	1.00	2.40	10000.00
18	12	Block 1	45.00	1.00	2.40	10000.00
23	13	Block 1	35.00	1.00	2.40	10000.00
10	14	Block 1	40.00	0.50	1.50	15000.00
2	15	Block 1	40.00	0.50	1.50	5000.00
8	16	Block 1	40.00	1.50	3.30	5000.00
25	17	Block 1	35.00	1.00	2.40	10000.00
5	18	Block 1	30.00	0.50	3.30	5000.00
11	19	Block 1	30.00	1.50	1.50	15000.00
6	20	Block 1	40.00	0.50	3.30	5000.00
24	21	Block 1	35.00	1.00	2.40	10000.00
26	22	Block 1	35.00	1.00	2.40	10000.00
14	23	Block 1	40.00	0.50	3.30	15000.00
15	24	Block 1	30.00	1.50	3.30	15000.00
28	25	Block 1	35.00	1.00	2.40	10000.00
21	26	Block 1	35.00	1.00	0.60	10000.00
1	27	Block 1	30.00	0.50	1.50	5000.00
17	28	Block 1	25.00	1.00	2.40	10000.00
22	29	Block 1	35.00	1.00	4.20	10000.00
13	30	Block 1	30.00	0.50	3.30	15000.00

- **Sterilization of prepared nanoparticles**

Prepared HBsAg loaded nanoparticles were sterilized by sterile filtration method. This method of sterilization is not affects the physicochemical properties of the nanoparticles. In this process, freshly prepared HBsAg loaded nanoparticles were filtered through membrane filter (0.22 µm, Merck Life Science Pvt Ltd., Bengaluru, India) with the help of needle and syringe [Li *et al.*, 2013].

- **Freeze drying of prepared nanoparticles**

HBsAg loaded nanoparticles were freeze dried by adding suitable and convenient cryoprotectant (mannitol 2% w/v) and following an optimized freeze dried cycle. The condenser temperature was maintained at -45°C and pressure applied in each step in shown in Table 4.3. The freeze drying process follows mainly three steps such as

(I) Freezing

(II) Primary drying

(III) Secondary drying.

Finally all vials were subjected to freeze drying process. Freeze-dried nanoparticles were analysed by normal observation of the volume of final sample and appearance of cake. Most important desired characteristics of freeze-dried nanoparticles contain an intact cake occupying the same volume as the original frozen mass. For determination of reconstitution time, rehydrate the freeze-dried nanoparticles with the similar volume of water lost after lyophilisation [Danhier *et al.*, 2010]. Generally, freeze dried nanoparticles are easily and immediately rehydrated after the addition of water but some time it require longer reconstitution time and thus there is chance of degradation of

formulation. Various methods have been developed for the re-suspension of freeze dried nanoparticles such as sonication or vortexing and manual shaking.

Table 4.3: A freeze drying cycle for antigen loaded polymeric nanoparticles

Freeze drying	**Steps**	**Temp. (ºC)**	**Time (Min)**	**Vacuum (mTorr)**	**Ramp/Hold**
Thermal Treatment	Step 1	25	15	-	H
	Step 2	0	30	-	R
	Step 3	0	60	-	H
	Step 4	-30	60	-	R
	Step 5	-30	60	-	H
Extra freezing steps	Freeze Temp. -55 Additional Freeze time 120 min Condenser set point -45 °C Vacuum set point 500 mTorr				
Primary drying	Step 1	-40	120	200	R
	Step 2	-40	240	200	H
	Step 3	-20	120	100	R
	Step 4	-20	240	100	H
	Step 5	0	120	50	R
	Step 6	0	600	50	H
	Step 7	25	120	20	R
	Step 8	25	120	20	H
Secondary drying	Step 1	25	150	50	H

4.2.3 *In-vitro* characterization of polymeric HBsAg loaded nanoparticles

- **Particle size, polydispersity index and zeta potential**

The average particle size was measured by determining the random changes in intensity of light scattered by suspended nanoparticles during their brownian motion. Polydispersity index indicates the distribution of nanoparticles which showed the nature of particles distribution in media like monodisperse and polydisperse. Zeta potential was determined by movement of charged nanoparticles under an applied electric field from Doppler shift of scattered light. It is based on the Helmoltz-Smoluchowski equation [Asadi *et al.*, 2013]:

$$\xi = Ue \times 4\pi\eta/\varepsilon$$

Where,

ξ is zeta potential, Ue is electrophoretic mobility, η is viscosity and ε is dielectric constant of the medium.

The average particle size (Z-average), polydispersity index (PDI) and zeta potential of the nanoparticles was determined by Beckman, USA Coulter particle size analyser after re-suspension of the prepared freeze-dried nanoparticles at 25 °C. Nano suspensions were filled in polystyrene sample cuvette and 1 mg/mL concentrated sample was prepared in milli-Q water sonicated on an ice bath. All measurements were performed in triplicate and represented as mean ± standard deviation. Similarly, for the zeta potential measurement, nano suspensions were filled in capillary tube of zeta cell and measured in triplicate.

- **Entrapment efficiency**

The entrapment efficiency was determined by using an in-direct procedure. In this method, 2 mL of prepared nanoparticle suspension was centrifuged at 18,000 rpm for 15 min room temperature. An aliquot of 100 µL supernatant was collected, and further diluted. Sample was prepared in triplicate and analysed by RP-HPLC method. The following equation was applied for the estimation of antigen entrapment:

$$\text{Entrapment Efficiency} = \frac{\text{Initial amt. of antigen} - \text{drug in supernatant}}{\text{Initial amt. of antigen}} \times 100$$

4.2.4 Surface characterization

4.2.4.1 SEM (Scanning Electron Microscopy)

Surface morphology of the antigen loaded PLGA nanoparticles were evaluated by SEM (Hitachi High Technology, Pleasanton). Smear were prepared by spreading the nano suspension on cover slip, after air drying particles were coated with a tinny layer of carbon and then examined under scanning electron microscopy.

4.2.4.2 TEM (Transmission Electron Microscopy)

It is common to use a negative staining with 2% w/v solution of phosphotungstic acid. TEM of HBsAg loaded nanoparticles was performed by negative staining. Briefly, the nanoparticles (20 mg/mL) dispersion was sonicated on water bath for 10 minutes. The internal morphology of nanoparticles was studied by putting 10 µL of nanoparticles suspension on copper grids followed by air-drying. Nanoparticles images were taken on

transmission electron microscopy (Tenai G2 20 Twin, FEI Company, Netherland) at All India Institute of Medical Sciences, New Delhi.

4.2.4.3 AFM (Atomic Force Microscopy)

The external morphology of HBsAg loaded nanoparticles was further visualized by Scanning probe microscope (NTEGRA Prime, NT-MDT) in semi contact mode. It having three mode tapping mode, contact mode and semi contact mode. In tapping mode probe was tapped to surface while in contact mode probe was moved in two direction, width wise and lengthwise. One drop of nanosuspension was placed on the small microscope slide to form a dry film of suspension for observation. Image was captured to determine average height and average roughness.

4.2.5 *In-vitro* release of HBsAg from nanoparticles

The release of HBsAg (mol. Wt. 10.0-12.0 kDa) from shell of PLGA was performed in phosphate buffer saline at pH 7.4 using dialysis bag method (DM). It is the most versatile and popular indirect method which allows ease of sampling at periodic intervals. 2 mL of nanoparticles solution were introduced into a dialysis bag (Hi-media, molecular weight 12,000-14,000 Da) and sealed properly. Bag was placed in a larger container having 100 mL of phosphate buffer saline (pH 7.4) at 37 ºC, under gentle agitation [Chisari *et al.,* 2010]. 0.05% sodium azide was added into the buffer as a preservative to prevent the growth of bacteria. Antigen released from the nanoparticles through the dialysis membrane to the outer release media compartment from where it was sampled for analysis by diffusion. The sample were withdrawn at 0 to 36 days, the intervals were taken in 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 2 days to 36 days. An aliquot of (1 mL) sample was collected and equivalent volume of fresh buffer was

replaced and further sample was processed for RP-HPLC analysis. All samples were prepared in triplicate. The data was analysed using linear regression equations and the order of drug release from the nanoparticles suspension was determined (zero order, first order, Higuchi diffusion model and Korsmeyer-Peppas model).

- **Zero order kinetics:** The rate is apparently independent of the reactant concentration. The ratio of absolute cumulative released amounts of drug at time t (Mt) and infinite time (M∞), i.e. in vitro fraction release profiles (Ft) is directly proportional to the time t can be donated by following equation:

$$Mt/M\infty = k_0 t$$

$$Ft = k_0 t$$

Where k_0 is the zero order release constant

- **First order kinetics:** Depends on the concentration of only one reactant amount. This model explain the absorption or elimination of few drugs, also describe the dissolution profile of pharmaceutical dosage forms those having water-soluble drugs in porous matrices. The release of the drug that follows first order kinetics can be expressed as:

$$dC/dt = - Kc$$

Where K is rate constant

Again equation can be written as:

$$\log C = \log C_0 - Kt / 2.303$$

Where C is the initial concentration of drug, C_0 is concentration at the time t, k is the rate constant, and t is the time. The data obtained are plotted as log cumulative percentage of drug remaining vs. time which results found straight line with a slope of -K/2.303.

- **Higuchi model (1963):** It gives several aspects of release of low soluble drugs incorporated in solid and semi-solid matrixes. Mathematical expressions were obtained for drug particles uniformly dispersed in homogeneous matrix which act as diffusion media. The Higuchi model expressed as:

$$Ft = Q = A \sqrt{D(2C- Cs)\, Cs\, t}$$

Where Q is the quantity of released drugs at the time t in per unit area A, C is the drug initial concentration, Cs is the drug solubility in the matrix media and D is the diffusion coefficient in the matrix substance. In a general way it is possible to simplify the Higuchi model expressed as:

$$Ft = Mt/M\infty = k \sqrt{t}$$

Where k denotes the release rate constant and model assumes that Fickian diffusion is the rate limiting step as well as shows the predominant release mechanism.

- **Korsmeyer-Peppas model (1983):** A simple equation which explained drug release from a polymeric system. When drug is released from matrix, the first 60% drug release data were fitted in Korsmeyer- Peppas model

$$Mt / M\infty = kt^n$$

Where,

Mt/M∞ is fraction of drug released at time t, k is the release rate constant and n is the release exponent. The n value is used to characterize different release for cylindrical shaped matrices.

4.2.6 Structural integrity of HBsAg loaded nanoparticles

The integrity of HBsAg in the polymeric nanoparticles shell was identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and compared with reference markers. It is based on the movement of charged particles in applied electric field. The molecules migrates, when placed an electric field, the rate of migration of sample molecules depends upon its size, shape, net charge and applied electric currents. It can be expressed as:

$$V = (E*q)/f$$

Whereas: V= velocity of migration of the charge molecules

E= electric field in volts per cm

q= net electric charge of the molecules

f= frictional coefficients

HBsAg was extracted by dissolving the nanoparticles in 2 mL of 5% (W/V) SDS in 0.1M sodium hydroxide solution. Obtained antigen was loaded onto a stacking gel and subjected to electrophoresis on a 12% separation gel at 200V until the dye band reached the gel bottom. After migration of samples, gel was stained with Coomassie blue dye to reveal the antigen, which was then, de stained and dried [Chisari *et al.*, 2010].

4.4.7 *In-vitro* cellular uptake study of nanoparticles

The cellular uptake study was performed in isolated human macrophage cells and MRC-5 human fetal lung fibroblast cell line. Macrophage cell was isolated and cultured in Department of Zoology, Banaras Hindu University, Varanasi, India and MRC-5 Cell line is obtained from National Centre for Cell Science (NCCS), Pune, India.

The following procedure was used for the uptake of nanoparticles:

- The isolated cells were culture and treated with 100 µL of FITC (fluorescein isothiocyanate) conjugated HBsAg loaded nanoparticles for 24 h.
- Cells were washed with ice cold PBS and again added 0.1% PBS and 0.1% NaN_3 solution. Again, cells were treated with 4% para-formaldehyde solution for 30 min and again washed with PBS buffer having 0.3% Triton X-100.
- Untreated cells were used as control and referred for comparison.
- After 24 h, cells were properly washed with phosphate buffer saline (pH 7.4) and observed in microscope.
- Nikon E800 upright fluorescence microscope equipped Hoechst 33258 and FITC with PE filter (with EXI aqua camera and NIS element software) was used for visualizing the images. Images were taken with 100X oil immersion objective lens in spectral range from 350-461 nm.

The uptake kinetic of prepared nanoparticles was demonstrated by using fluorescence technique. Immuno fluorescence uptake kinetic was performed in macrophage cells for 24 hr. 100 µL of fluorescent marker loaded (FITC) conjugated blank nanoparticles, plain HBsAg, alum adsorbed HBsAg, HBsAg loaded nanoparticles were added to wells of culture plate. Uptake kinetic study of cultured cell was studied at predetermined time

intervals such as 0, 0.5, 1, 2, 4, 8, 16 and 24 hr. Cells were harvested and excess of formulation was separated by washing with ice-cold PBS at the end of each interval. After washing, cells were again added in 0.1% PBS and 0.1% NaN_3. These cells were analysed by flow cytometry (Coherent, Palo Alto, CA) equipped with an Innovate 90-5 argon ion laser light working at 488 nm and 515 mW wave length [Jaganathan and Vyas, 2006].

4.2.8 Haemocompatibility studies

4.2.8.1 Evaluation of haemolysis

Evaluation of haemolysis is essential to prove haemocompatibility when the nanoparticles formulations were intended for intra muscular administration. Haemolysis study of placebo-nanoparticles, plain HBsAg, alum HBsAg and HBsAg loaded nanoparticles were studied using earlier reported method [Mishra *et al.*, 2010; Hung *et al.*, 2004]. Briefly, Human blood was procured from an authorized blood bank. Plasma layer was decanted carefully and added normal saline solution in same volume and mixed softly. Sample (5 mL) was taken in a sterile graduated centrifuge tube and centrifuge in 1344×g for 10 min at room temperature. Again erythrocyte suspension was centrifuged. The same procedure was repeated three times for washing. Finally, diluted up to 50 mL by normal saline solution and re-suspended.

Sample like plain HBsAg, HBsAg loaded nanoparticles and alum HBsAg (equivalent to 10, 20 and 30 ng/mL of HBsAg), placebo-nanoparticles (equal to volume of HBsAg loaded nanoparticles) were added separately in sterilized tubes containing 2 mL of erythrocyte suspension. 100% lysed erythrocytes used as positive control and same volume of diluted normal saline having erythrocyte suspension with 1% triton X-100

were used as the spontaneous negative control, respectively. Prepared samples were incubated at 37 ºC for 30 min and mixed gently in every 15 min. 200 µL sample aliquots were collected at 0.5, 1, 2, 4 and 8 hr interval then centrifuged at 1344×g for 10 min. For the oxidation of haemoglobin to oxy-hemoglobin 100 µL supernatants were withdrawn from sample and incubated for 30 min at room temperature. Absorbance was measured spectrophotometrically at 595 nm (Synergy H1 hybrid, Biotek, USA). Experiments were performed in triplicate and expressed the data as mean± SD (n=3). Haemolysis percentage was calculated by following formula:

$$\% \text{ Haemolysis} = \frac{A_{\text{Sample}} - A_{\text{Spontaneuous Control}}}{A_{\text{Positive Control}}} \times 100$$

Where, A_{sample} is the absorbance of supernatant of erythrocytes incubated with test sample, $A_{Spontaneous\ Control}$ is the absorbance of supernatant of erythrocytes incubated with normal saline and $A_{Positive\ Control}$ is the absorbance of supernatant of erythrocytes incubated with 1% triton X-100 solution.

4.2.8.2 Quantitative platelet aggregation evaluation

Aggregation afterward treating with placebo-nanoparticles, plain HBsAg, HBsAg loaded nanoparticles and alum HBsAg was performed [Mishra *et al.,* 2010; Hung *et al.,* 2006]. Platelet aggregation quantitatively is performed using haematological counter (Multisizer 4, Beckmann Coulter, USA) after the test samples were incubated with blood sample. Briefly, plain HBsAg, HBsAg loaded nanoparticles and alum HBsAg (equivalent to 10, 20 and 30 ng/mL of HBsAg). Placebo nanoparticles were taken to equal volume of HBsAg loaded NPs and incubated at 37 ºC for 2 hr with 1 mL of blood. Likewise, whole blood samples with PBS were used for spontaneous control at the same experimental conditions. Again 10 µL samples were diluted up to 10 mL with isotome,

mixed and analysed by haematological counter. All experiment and sample analysis were performed in triplicate and final results were expressed as mean± SD (n=3).

4.2.8.3 Qualitative platelet aggregation

Qualitative platelet aggregation performed by microscopic evaluation of stained peripheral blood smears after incubating the whole blood with test samples. Samples were prepared by after incubation of peripheral blood smears. Samples were spread on properly cleaned glass slide and further air dried followed by Leishman's staining for 5 min (Span Diagnostics, India). After rinsing the slides, placed cover glass on it and analysed on optical microscope contain is immersion objective and images were captured by digital camera.

4.2.9 Stability study

The stability of HBsAg loaded PLGA nanoparticles, especially in their core-environment, was investigated. For this purpose lyophilized HBsAg-PLGA nanoparticles were incubated at 30 ± 2^{0}C (R.H. 65±5%, n=6) for 90 days. A sample was withdrawn at each time-point (30, 60 and 90 days) and evaluated for changes in particle size, zeta potential and encapsulation efficiency [Jaganathan *et al.,* 2006; Pawar *et al.,* 2010].

4.2.10 Selection of route of administration in BALB/c mice

- **Animals and study design**

BALB/c male mice 6–8 weeks age were used in all experiments. Animals were kept in four group and each groups had six (n = 6) animals and allowed free access of food and water. At least 3 h before immunization all food materials were withdrawn. The protocol was approved by Institutional Animals Ethical Committee, Indian Institute of Medical Science, Banaras Hindu University, Varanasi, India (Dean/2015/CAEC/1424). Studies were conducted as per the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. To induce an immune response, single dose of 10 µg HBsAg nanoparticles in phosphate buffer saline was administered with different routes like oral, sub-cutaneous, nasal and intramuscular. Table 4.4 shows the *in-vivo* immunization schedule used in the experiment. The blood samples were collected from retro-orbital plexuses during and post-immunization at 2, 4, 6 and 8 weeks for estimation of anti HBsAg studies. All the collected blood samples were centrifuged to separate serum and serum were stored at -20ºC until analysed.

The mice were immunized by 10 µg of intragastric administration of HBsAg nanoparticles formulation. For the nasal route antigen loaded nanoparticles were administered intranasally in drops by using sterile syringe under 0.2 cm into the nostril. The mice were not anesthetized for dosing intranasally and the tests samples were direct by administered into nasal cavity in supine position [Iqbal *et al.,* 2003]. HBsAg nanoparticles formulation was also administered subcutaneous and intramuscularly. [Zambaux *et al.,* 1998].

Table 4.4: *In-vivo* immunization schedule used in experiment

S. No	Groups	No. of Mice	Formulation*	Route of Administration
1	A	6	10 μgHBsAg NPs	Nasal
2	B	6	10 μgHBsAg NPs	Sub-cutaneous
3	C	6	10 μgHBsAg NPs	Oral
4	D	6	10 μgHBsAg NPs	Intra muscular

* To induce an immune response, 10 μg dose of antigen is sufficient [Jaganathan and Vyas, 2006].

- **Blood sample collection**

After single dose administration of HBsAg nanoparticles, mice blood sample was collected in every 2, 4, 6 and 8 weeks by retro-orbital puncture and immunoglobulin IgG was estimated. After 4 and 8 weeks immunoglobulin IgA was estimated. Endogenous cytokines levels such as interferon-*Y* (IFN-*Y*) and interleukin-2 (IL-2) was measured in last week of experiment. Serum sample was stored at −20 ºC till analysed by ELISA.

- **Measurement of immunoglobulin (IgG and IgA) response**

Immunoglobulin IgG produced against the administration of the HBsAg, immunoglobuline was measured in blood serum sample through solid-phase enzyme-linked immunoassay kit [Mishra *et al.,* 2006].

The ELISA microtiter 96 well plates (Nunc-Immuno plate) were coated with 100 μL solution of HBsAg in10 μg/mL in PBS, pH 7.4 and incubated for overnight at 4 ºC. Plate was washed three times with PBS-T (0.05% v/v Tween 20 in PBS). Serial

dilutions of samples in 1% (w/v) PBS–BSA were added in each well then incubated the plate for 2 hr at room temperature and again washed three times with PBS-T followed by 100 μL of diluted horseradish peroxidase conjugated goat antimouse-IgG. After that in place of horseradish peroxidase conjugated goat antimouse-IgG added IgA in each well and incubated for 2 hr. Further plate was washed three times with PBS-T and then added substrate solution (3, 3, 5, 5-tetramethyl benzidine containing hydrogen peroxide) in each well, incubated under dark place at room temperature for 15 min and then added 50 μL of H_2SO_4 (2 M) in each well to stop the colour reaction and measured the absorbance at 491 nm in ELISA microplate reader. The concentration was found in mIU/mL and standard calibration curve was prepared. Antibody levels were plotted as log of anti-HBsAg antibody titres (mIU/mL) versus time in days [Debin *et al.*, 2002].

- **Estimation of cytokines (IFN-Y and IL-2) levels**

Separate ELISA kits were used for the cytokines endogenous levels of IL-2 and IFN-*Y*. As per manufacturer protocol the blood serum cytokines levels were estimated by ELISA method in the last week of experiments *i.e.* 8^{th} week. The double antibody sandwich ELISA was used for the estimation. It provides antigen capture and immune specificity, whereas another antibody which is linked to an enzyme help in detection and amplification.

- **Statistical analysis**

Statistical analysis was achieved on the basis of *in-vivo* data followed by one-way analysis of variance (ANOVA) with applied Tukey-Kramer multiple comparisons post-test using Graph Pad Prism 5 Software and a significance levels less than 0.05 ($p<0.05$) was selected.

4.2.11 *In-vivo* cellular internalization study

Female BALB/c mice 6–8 weeks old were used and given free access to food and water. They were denied of any food intake 3 hr before immunization. The animal study protocol approved by Institutional Animal Ethical Committee of Indian Institute of Medical Science, Banaras Hindu University, Varanasi, India (Dean/2015/CAEC/1424). was followed. The studies were carried out as per the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

The uptake behaviour and imaging of nanoparticles was demonstrated by using fluorescence technique. The following procedure adopted for internalization of HBsAg loaded nanoparticles:

- BALB/c mice were taken and fluorescent marker loaded (FITC) nano formulations were administered by intramuscular injection route.
- After 2 hr animals were sacrificed and lymph nodes and spleen were separated out.
- Lymph nodes and spleen was preceded for microtomy study.
- 5 μm thickness sections were viewed under fluorescent microscope, (Nikon Eclips E 600, with ECD equipped video camera) and image was captured.

4.2.12 Immunological characterization and measurement of antibody levels

- **Animal and study design**

In all animal experiments female BALB/c mice 6–8 weeks old were used. Mice were grouped in six (*n*=6) with given free access to food and water. They were denied of any food intake 3 h before immunization. The animal study protocol approved by Institutional Animal Ethical Committee of Indian Institute of Medical Science, Banaras Hindu University, Varanasi, India (Dean/2015/CAEC/1424). was followed. The studies were conducted as per the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Induction of an immune response, 10 µg antigens was inoculated intra muscularly (I.M.). Table 4.5 represent the various formulations administered in mice.

Table 4.5: *In-vivo* immunization schedule used in experiment

S. No	Groups	No. of mice	Formulation*
1	A	6	Normal saline
2	B	6	Alum adsorbed HBsAg single dose
3	C	6	Alum adsorbed HBsAg booster dose
4	D	6	Normal antigen
5	E	6	Nanoparticles single dose
6	F	6	Nanoparticles booster dose

* To induce an immune response, 10 µg dose of antigen is sufficient [Jaganathan *et al.*, 2006].

After 4 weeks secondary immunization was done with the equivalent dose of formulations. The control group, is given an intra-muscular 10 µg dose of alum-adsorbed HBsAg (alum used as adjuvants) and booster dose given after four weeks of

primary immunization. Second group received single dose of alum-adsorbed HBsAg. The third and fourth group received normal saline and booster dose of normal antigen respectively. Another two groups (*i.e.* 5 and 6^{th}) received antigen loaded polymeric nanoparticles in single and booster dose, respectively.

- **Sample collection**

Blood serum sample was collected at after 2, 4, 6 and 8 weeks by retro-orbital puncture in mice. The immunoglobulin IgG was estimated after 2, 4, 6 and 8 weeks whereas immunoglobulin IgA was estimated after 4 and 8 weeks. Endogenous cytokines levels such as interferon-*Y* (IFN-*Y*) and interleukin-2 (IL-2) was measured in last week of experiment. Serum sample were stored at -20 ºC until analysed by enzyme-linked immunosorbent assay (ELISA).

- **Measurement of immunoglobulin (IgG and IgA) response**

Immunoglobulin IgG produced against the administration of the HBsAg, immunoglobuline was measured in blood serum sample through solid-phase enzyme-linked immunoassay kit [Mishra *et al.,* 2006].

The ELISA microtiter 96 well plates (Nunc-Immuno plate) were coated with 100 µL solution of HBsAg in10 µg/mL in PBS, pH 7.4 and incubated for overnight at 4 ºC. Plate was washed three times with PBS-T (0.05% v/v Tween 20 in PBS). Serial dilutions of samples in 1% (w/v) PBS–BSA were added in each well then incubated the plate for 2 hr at room temperature and again washed three times with PBS-T followed by100 µL of diluted horseradish peroxidase conjugated goat antimouse-IgG. After that in place of horseradish peroxidase conjugated goat antimouse-IgG added IgA in each well and incubated for 2 hr. Further plate was washed three times with PBS-T and then

added substrate solution (3, 3, 5, 5-tetramethyl benzidine containing hydrogen peroxide) in each well, incubated under dark place at room temperature for 15 min and then added 50 μL of H_2SO_4 (2 M) in each well to stop the colour reaction and measured the absorbance at 491 nm in ELISA microplate reader. The concentration was found in mIU/mL and standard calibration curve was prepared. Antibody levels were plotted as log of anti-HBsAg antibody titres (mIU/mL) versus time in days [Debin *et al.*, 2002].

- **Estimation of cytokines (IFN-Y and IL-2) levels**

Separate ELISA kits were used for the cytokines endogenous levels of IL-2 and IFN-*Y*. As per manufacturer protocol the blood serum cytokines levels were estimated by ELISA method in the last weeks of experiments *i.e.* 8^{th} week. The double antibody sandwich ELISA was used for the estimation. It provides antigen capture and immune specificity, whereas another antibody which linked to an enzyme help in detection and amplification.

- **Statistical analysis**

Statistical analysis was achieved on basis of the in vivo data followed by one-way analysis of variance (ANOVA) with applied Tukey-Kramer multiple comparisons post-test using Graph Pad Prism 5 Software. All through, a significance levels less than 0.05 ($p<0.05$) was selected.

4.2.13 *In-vivo* Lymphocyte and T cells proliferation study

Lymphocyte proliferation assay is mainly related to the cell-mediated immune response and used for the determination of lymphocyte stimulation. Lymphocyte B and T cells proliferation leads to clonal expansion and it is the initiation of the specific immune response.

4.2.13.1 Lymphocyte proliferation test in BALB/c mice

Female BALB/c mice 6–8 weeks old were used with given free access to food and water. They were denied of any food intake 3 hr before immunization. The animal study protocol approved by Institutional Animal Ethical Committee of Indian Institute of Medical Science, Banaras Hindu University, Varanasi, India was followed. The studies were accepted as per the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Induction of an immune response, HBsAg loaded nanoparticles was administered intra muscularly and after four weeks secondary immunization was done with the equivalent dose of formulations. The experiment starts after the secondary immunization and followed the following procedure:

- Spleens were aseptically removed. Spleen was homogenized and made a complete suspension by passing through 100 µm nylon filter strainer. Suspension was treated with RBC lytic buffer solution to lyse the erythrocytes cells.
- The contaminated cells were removed by three washing of buffer solution and pure cells were suspended in RPMI Medium 1640 (Thermo Fisher Scientific) and 10% Fetal calf serum, then ELISA was performed.

- ELISA micro plate containing 96-well flat bottom plate received 100 µL prepared cells, added 100 µL of complete medium with concanavalin A (10 µg/mL) in each well.
- Finally test samples were added in culture plate and incubated for 72 hr at 37ºC in a humidified atmosphere with 5% CO_2.
- After that the cells were labelled with radioactive substance thymidine (1µci/well).
- Radioactive incorporated media was calculated by liquid scintillation-beta counter (Scimens, E-Cam, Germany).
- The proliferative response results are expressed as a stimulation index (SI), which is calculated by dividing the mean counts per minute (cpm) of mitogen-stimulated wells by the mean cpm of non-stimulated wells.

4.2.13.2 T cell proliferative study in human peripheral blood

For the long lasting immunity, T cells play an important role because it regulates both cellular and humoral immunity. When foreign antigen enters into the body, immune systems immediately recognize and generate immune response. If this immunity is due to involvement of T-cells, immunological T-cells memory is generated. When body encounters the same antigen produce stronger immune response because of existing of immunological memory. The principle of the immunization is priming and booster. Priming is the first antigenic stimuli, whereas booster is the subsequent antigenic stimuli [Zhao *et al.*, 1996; Singh *et al.*, 2002]. For this purpose, isolated T cells were cultured in medium containing different samples. T cells were isolated in human blood sample. The human blood sample was procured from an authorized blood bank and following procedure was followed for isolation of T cells:

- Human peripheral blood was used for extracting mononuclear T cells by Ficoll density gradient centrifugation method.
- Extracted T cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific) having autologous serum for one hour. Nylon wool separation method was used for isolation purification of T cells.
- These purified T cells were cultured in medium containing blank nanoparticles, plain HBsAg, alum adsorbed HBsAg and HBsAg loaded nanoparticles for 6 days.
- BrdU (5-bromo-2-deoxyuridine) incorporation method was used to measure proliferation of T- cell. BrdU is a synthetic nucleoside which is an analog of thymidine.
- Again cultured cells were extracted and washed with PBS. Cells were labelled with anti-BrdU monoclonal antibody-FITC-conjugate (BD Biosciences) and again dual labelling with anti-CD3-monoclonal antibody-PE (Phycoerythrin) conjugated (EXBIO Antibodies).
- Flow cytometer cells analyser FACS Calibur (BD Biosciences, USA) equipped with an Innovate 90-5 argon ion laser and operating at 488 nm with 515 mW light-regulated mode was used for assay and measurement of T-cell proliferation response.

4.2.14 Assessment of immunological parameter in Humanized Xenograft model

4.2.14.1 Development of Humanized Xenograft model

- **Animal and study design**

BALB/c female mice of 6 to 12 weeks of age were obtained from Zoology Department, Banaras Hindu University, Uttar Pradesh, India. BALB/c mice were lethally irradiated by specific protocol. In our study these mice is also called as recipient mice.

NOD/scid mice 4 to 8 weeks old obtained from Centre of Cellular and Molecular Biology Hyderabad, India. Bone marrow was obtained from these mice and transplanted into recipient BALB/c mice though intravenous injection with phosphate-buffered saline (PBS). The main region of selection of NOD/scid mice is, due to lack of functional B cells and T cells but also low macrophage activity and reduced natural killer (NK) cell. The schematic representation of bone marrow component is shown in Figure 4.1.

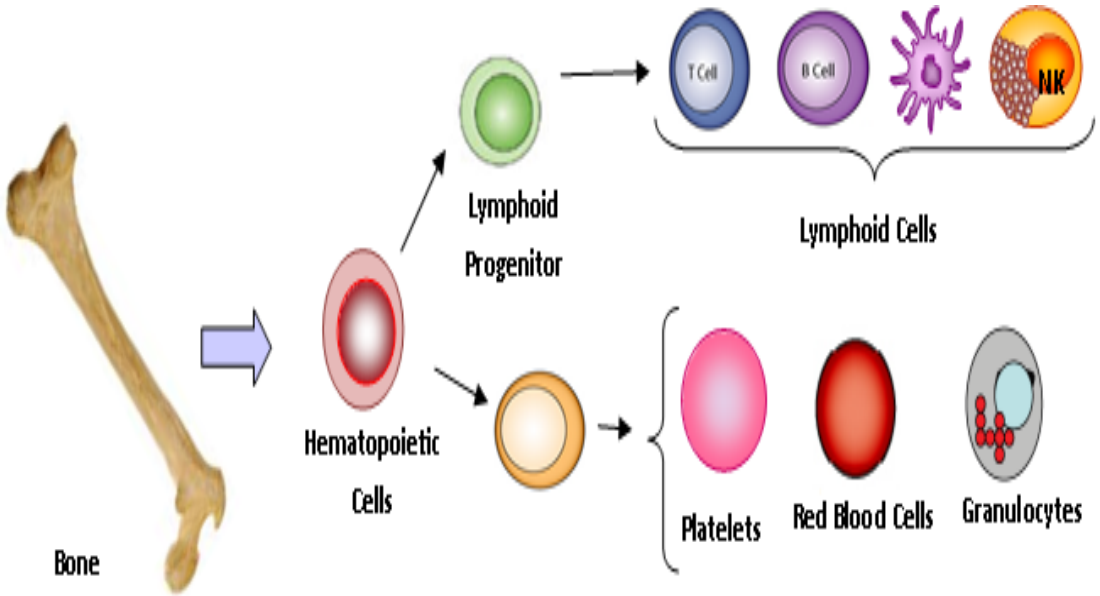

Figure 4.1: Component of bone marrow cells

All mice were kept under animal ethical committee protocol like pathogen free environment, given sterile food, and provide acid water having ciprofloxacin (20 μg/mL). Ciprofloxacin is an antibiotic in a group of drugs called fluoroquinolones. Ciprofloxacin fights against the pathogens and protect body from bacterial infections.

- **Irradiation of recipient's mice**

The successful survival of a bone marrow graft depends upon suppression of the host's immune system. Irradiation process causes depletion of the bone marrow function of the host progenitor cells. It provides a complete space for engraftment of donor stem cells. For this purpose ionization radiation was used for suppression of the host immune system. Ionizing radiation causes break in DNA double-strand at multiple sites. For the ionization process gamma irradiation are mostly used. The whole-body gamma irradiation is commonly accomplished in small animals. Before irradiation mice had given only sterile feed with medicated water. Medicated water contains:

- Sulfamethoxazole (200 mg/mL) and trimethoprim oral suspension (40 mg/mL) added to 8 gallon acidified water bottle (3.4 mL per bottle) given every other week for dose of 40mg/8mg per kg. (1mg/0.2mg per 25g mouse).
- Animal water acidified was maintained by sulfuric acid to pH 2.4 to 3.1.

The amount of irritation is limited (300- 700 cGy) for mice at very short period around seven minutes because mice are very sensitive to radiation. The irradiator chamber is small size, which holds only a few mice at a time [Duran-Struuck *et al.*, 2009]. Gamma radiation should be given in two equal doses provide 2-3 doses apart. After radiation mice were taken back to cage and given medicated water, sterile feed and maintained properly.

- **Isolation of bone marrow from NOD mice**
  - Bone marrow was isolated from femur and tibia bone cavities. The donor mice were euthanized and pull both halves of the skin away from the incision.
  - During over all procedure 70% ethanol was sprayed periodically in mice, to maintain the aseptic condition in experiment.
  - Legs were taken out and hairs were remove. Thoroughly scrap the tibia and femur of all fascia, muscle and connective tissue. Epiphysis and distal ends of each bone was clipped.
  - Approximately 7 mL culture media was placed in a small culture dish and about 40 mL media in a large culture dish in the hood.
  - In 2 mL media bone marrow was flushed with a 27- gauge needle in to the culture dish.
  - The culture dish was rinsed and washed and the cells were collected in fresh media.
  - This culture cell is transplanted in BALB/c mice by I.V. administration, which was already lethally irradiated**.** Figure 5.2 represented the isolation of bone marrow cells from tibia and fibula

  - Materials and Reagents:
    - Culture media (5-10% fetal bovine serum RPMI)
    - Conical flask, test tubes
    - Tissue culture plates
    - Dissection tool and tray
    - 70% Ethanol

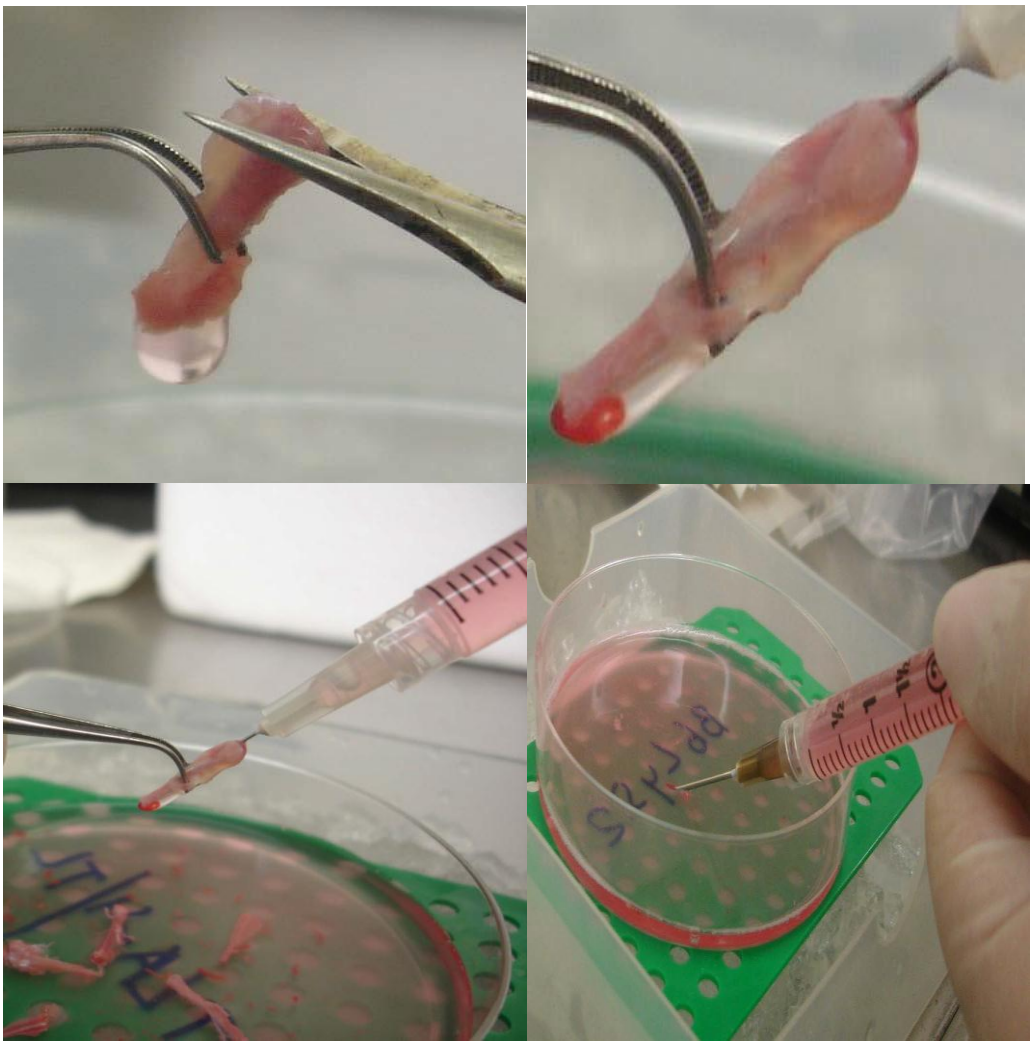

Figure 4.2: Isolation of bone marrow cells from tibia and fibula

- **Human blood donors and patients**

Many persons don't identify when or how they acquired the infection. According to previous study, about 15% of people who got infected with Hepatitis B are incapable to recognize a risk factor that explains why they have the disease. Only blood test is the method for the detection of infection. Mostly following three standard blood tests are performed:

(1) HBsAg (Hepatitis B surface antigen): The surface antigen is "positive" or "reactive," it means the person is now infected with Hepatitis B virus and is capable to infect the other person or pass the infection on to others.

(2) Anti-HBs [HBs-Ab] (antibody to Hepatitis B surface antigen): When this is "positive" or "reactive," means person is immune to Hepatitis B infection, either from past infection or from vaccination.

(3) Anti-HBc [HBc-Ab] (antibody to Hepatitis B core antigen): When anti-HBc is "positive" or "reactive," it might mean the person has had now contact with virus. Actually it is very complicated test to explain because the "anti-HBc" can possibly be a "false-positive" test result.

(4) The fourth blood test is done is IgM anti-HBc (IgM class antibody to Hepatitis B core antigen). If it is positive or "reactive," means that the person has suffered from Hepatitis B infection in the past six months, showing acute (recently acquired) condition of Hepatitis B infection.

The infected human peripheral Blood Mononuclear Cells (PBMCs) were obtained from HBV infected/ immunized human donors (blood sample obtained from Parul Patholab, Varanasi, Uttar Pradesh), which having more than six month after spontaneous clearance of HBV infection i.e. donors blood having positive for HBsAg, HBcAg and anti-HBe antibodies (Table 4.6).

Donors used in experiments were completely healthy and negative for anti-human immunodeficiency virus (HIV)-1/2 antibodies or anti-Hepatitis C virus (HCV).

Table 4.6: Serological data of PBMCs Donors/patients suffering from HBV

Anti-HBs (mU/ml)	**Anti-HBc**	**Anti-HBe**	**HBsAg**	**HBcAg**	**HBeAg**
-- (-Ve)	-- (-Ve)	0.04 (+Ve)	-- (+Ve)	-- (+Ve)	0.27 (-Ve)
HBV-DNA (mIU/ml)	**AST/ALT (U/L)**	**Bilirubin (mg/dl)**	**Prothrombin Time (Sec.)**	**Albumin (g/dl)**	
2186 (+Ve)	<1	0.2	7-8	4.4	

Peripheral Blood Mononuclear Cells (PBMCs) were obtained from more than six month of HBV infected/ immunized human donors. The Hepatitis B virus DNA was also present in blood sample and very high amount 2186 mIU/mL. Anti-HBs–secreting B cells and anti-HBs specific IFN-*Y* secreting T cells were found in the PBMC of donor blood in high frequencies. The Aspartate Aminotransferase and Alanine Aminotransferase were also measured in donor's blood. The ratio of AST (Aspartate Aminotransferase) and ALT (Alanine Aminotransferase) of patients' blood was found to be less than one; it indicates non-alcoholic fatty liver disease and viral hepatitis. Levels of bilirubin are 0.2 mg/dI and albumin levels is 4.4 g/dI.

- **Isolation and transplantation of human PBMCs**

Subsequently bone marrow transplantation (BMT) in recipients BALB/c mice, the human Peripheral Blood Mononucleolus Cell (PBMCs) was collected by leukapheresis, isolated through Ficoll density gradient centrifugation.

Reagents: - Phosphate buffer saline, pH 7.2

- 2 mM EDTA,

- 15 mL of Ficoll- Paque (Þ 1.077g/mL)

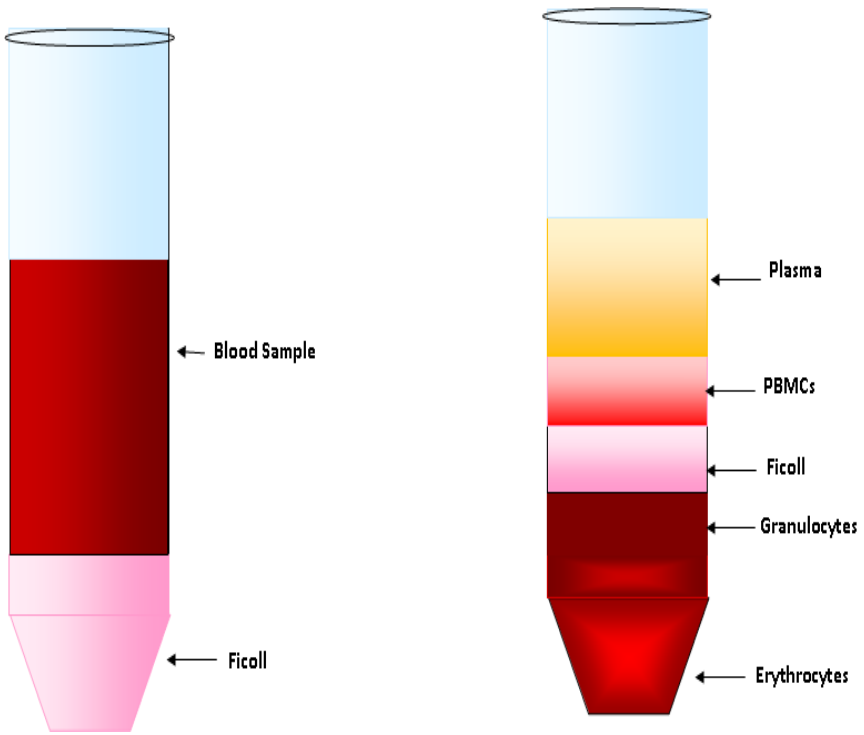

Figure 4.3: Mixture of diluted cells suspension in a Ficoll-Paque tube. After centrifugation found different layers of cells and blood component

PBMCs isolated from freshly withdrawn human blood and added with anticoagulants (EDTA), blood should not be older than 8 h. PBMCs cells were diluted with 2-4 times volume of buffer. Mixture of diluted cells suspension (35 mL) and Ficoll-Paque (15 mL) were prepared in 50 mL of conical tube. Prepared mixture were centrifuge at 400Xg for 30 min at 20 ºC and after that different layer of sample was found (Figure 4.3). Leaving the mononuclear upper cell layer and re-suspend the cell palate in buffer (50 mL) and centrifuge at 200Xg for 15 min at 20 ºC, carefully supernatant were completely removed. The following steps were repeated and properly collect the PBMCs. Separated cells were cultured in RPMI-1640 medium containing autologous serum. Finally 8 to $10X10^7$ PBMC cells were injected Intra-Peritoneally (IP) in recipient mice.

4.2.14.2 Study design and vaccination

After the successful transplantation and irradiation of BALB/c mice, vaccination was given. The mice were divided in three groups, each groups having six mice. On the same day of experiments, first group of mice (having bone marrow transplant and received human blood mononuclear cells) was vaccinated with 5 μg of prepared polymeric nanoparticles through I.M. (Intra Muscular) route and toxoid injection (Table 4.7) whereas the control group or second group received only saline solution. The third group of chimeric BALB/c mice received vaccination having only bone marrow transplant by I.M. route. In experiment using tetanus toxoid, it protects against this life-threatening disease and provides protection against tetanus in chimeric mice.

Table 4.7: Chimera mice groups and vaccination schedule

S.No.	Group	Group received	Vaccination
1.	Group I	Bone Marrow Transplant + PBMC	Vaccine
2.	Group II	Bone Marrow Transplant + PBMC + TT	Saline
3.	Group III	Bone Marrow Transplant + PBMC +TT	Vaccine

4.2.14.3 Sample collection and antibody response measurement

PBMCs transplanted chimeric mice were checked routinely by isolation of cells and serum test. All control and vaccinated groups of mice were bled from the retro-orbital puncher and scarified by cervical dislocation at time points (1, 6, 12, 18 days). Spleens were removed aseptically and homogenized to form the small fragment of spleen and clear single cell suspension was prepared. Lymphocytes were separated by Ficoll density centrifugation and the isolated cells were cultured on RPMI-1640 medium containing autologous serum. The RPMI-1640 media contain 1 μg/mL phytohemagglutinin (PHA), 10% FBS (Fetal Bovine Serum) and 1% penicillin/ streptomycin.

Finally, three colour flow cytometer CD45-fluoroscein isothiocyanate (FITC), CD4-phycoerythrin (PE) and CD3-peridinin chlorophyll protein (Per CP) were performed. All reagent and instruments are made by Becton, Dickinson and company, BD Bioscience. Also at the above designed time points HBV envelop antigen (HBeAg), surface antigen (HBsAg), HBs antibody (HBs-Ab) and genomic DNA in serum were monitored. Immunohistochemistry was also performed to observe the infection of HBV in mice [Bocher *et al.,* 1999; Bocher *et al.,* 2000].

- **Estimation of human antigen–specific B and T cells from Chimera mice**

Antigen specific anti-HBs blood cells T and B were quantified by very commercially and accessible solid-phase enzyme-linked immunoassay kit method [Mishra *et al.*, 2006]. For the estimation of anti-HBs immunoglobulin G (IgG)- secreting B cells, microtiter plates (Nunc-Immuno 96 well plate) were coated with 100 μL solution of HBsAg (10 μg/mL in pH 7.4, PBS) and incubated overnight at 4ºC. After that plates were washed three times with PBS-T (0.05% v/v Tween 20 in PBS). Serial dilutions of samples in PBS–BSA (1% (w/v) were prepared and added in each well.

Plates were again incubated for 2 hr at room temperature and washed three times with PBS-T followed by 100 μL of diluted horseradish peroxidase conjugated goat anti mouse-IgG and incubated for 2 h. Further plate was washed three times with PBS-T and then added substrate solution (3, 3, 5, 5-tetramethyl benzidine containing hydrogen peroxide) in each well, incubated under dark place at room temperature for 15 min and then added 50 μL of H_2SO_4 (2 M) in each well to stop the colour reaction and measured the absorbance at 491 nm in ELISA microplatereader (Novex ELISA Kits, Thermo-Fisher Scientific). The concentration was found in mIU/mL and standard calibration curve was prepared. Antibody levels were plotted as log of anti-HBsAg antibody titres (mIU/mL) versus time in days [Marcus *et al.*, 1995; Debin *et al.*, 2002].

- **Estimation of cytokines (IFN-*Y*) levels**

The estimation of cytokine-secreting T cells was performed by ELISA kit. Coating and detection were done by monoclonal antibody against the determination of cytokines endogenous levels like interferon-*Y* (IFN-*Y*). Blood serums were collected at given time point and estimate the cytokines by selected ELISA method.

Detection of immunoglobulin was assessed routinely using a standard quantitative sandwich enzyme linked immunosorbent assay (ELISA) technique (Biomedicals). Tetanus Toxoid antigen was bound on the surface of the microtiter plate. Patient diluted serum was pipetted into the wells of microtiter plate. After found the binding between the serum antibodies and immobilized Tetanus Toxoid antigen was takes place. Afterward one hour incubation, plates were rinsed and washed to remove excess of unbound material. Added the anti-human-immunoglobulin peroxidase conjugate reagents and incubated. Then substrate (TMB) solution was pipetted and further incubated for 30 min. After the colour reaction stop measured the absorbance spectrophotometrically at the wavelength of 450 nm. The concentration of the antibodies was directly proportional to the intensity of the colour [Liang *et al.*, 2013].

- **Detection of HBsAg/HBcAg by ELISA assay**

Actually Hepatitis B surface antigen (HBsAg) is a lipoprotein and detection of HBsAg in human blood serum or plasma indicate an ongoing growth of hepatitis infection, it may be either acute or chronic stage of infection. It not only diagnoses the virus infections, also monitor the disease progress as well as efficacy of antiviral therapy. Quantitative detection of HBsAg was measured by ELISA technique in vitro followed by manufacturer's protocol. The HBsAg specific ELISA (Abbott India Ltd) microtiter plate solid phase is made of polystyrene and wall is coated with mouse monoclonal antibodies (HBsAb). Presence of HBsAb was analysed by pre-coated HBsAg plate and established by HRP-labeled (horseradish conjugate) anti-mouse IgG. The lower limit of detection of HBsAg was 0.5 ng/mL. Different concentration of serum dilutions were prepared to find the values within the linear range. Serum having HBsAg were added to the anti-HBs antibody-coated wells together with peroxidise conjugated anti-HBs

antibody and sample was incubated. After incubation an antibody HBsAg-antibody-peroxidase complex was obtained in the wells. Microtiter plate was washed with PBS for removing of unbound material. A substrate solution TMB (3,3′,5,5′-Tetramethylbenzidine) were added in each wells and again incubated at 37±0.5 °C for 15 min. A development of the colour is directly proportion to the concentration of HBsAg bound to anti-HBs. The antigen antibody reaction was stopped by addition of sulphuric acid. Finally measured an optical density of developed colour which were read by photometer at 450 nm with a selected reference wavelength.

HBV core protein (HBcAg) was detection and visualized by immmunohistochemical Staining.A small section of liver tissues were fixed with 10% neutral buffered formalin and embedded in paraffin. Then washed or deparaffinised with water and section were incubated with primary antibodies (polyclonal rabbit anti-HBcAg; DAKO, Glostrup, Denmark). After that counter stained was done with hematoxylin and visualized by microscope [Yant *et al.*, 2000; Raney *et al.*, 2001].

- **Quantitation of viremia by Real-time PCR**

Viremia (HBV-DNA) were measured by purification and quantitation of encapsidated viral DNA by real-time PCR (QIAGEN, Hilden, Germany) method. The lower detection limits 1X 10^5 genome equivalents per mL [Huang *et al.,* 2006]. Extract the viral DNA from 100 µL of blood serum and a mixture containing 10 pmol of each oligonucleotide primer and 0.5 U of Taq polymerase in reaction buffer (10 mmol/L Tris-HCl [pH8.3], 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 0.01% [wt/vol] gelatin, 500 µmol/L [each] dATP, dGTP, dCTP, and dTTP).

Primers and probe design: The PCR primers and probe were designed in order to equally amplify all known HBV genotypes. In this way, an alignment with sequences of all HBV genotypes and sequences from Gen Bank was carried out. After this, PCR primers and Taq probe sequences were checked concerning base composition, melting temperatures, GC content, internal folding using Primer Express software.

The PCR primers and probe were designed in order to equally amplify all known HBV genotypes. In this way, HBV-specific primers oligonucleotide 1, sense (5'-GGA-GGC-TGT-AGG-CAT-AAA-TTG-GTC-TGC-GC-3') was used. After this, PCR primers and probe sequences were checked concerning base composition and oligonucleotide 2, antisense (5'-CCC-GAG-ATTGAG-ATC-TTC-TGC-GAC-GCG-GCG-ATT-GAG-ACC-3') was used. HBV DNA Assay was performed in accordance with the manufacturer's protocol. The numbering starts from the EcoRI site and PCR products were analysed on a 1.5% agarose gel [Murphy, 2012].

DNA extraction: For the isolation of HBV DNA from serum, the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) was used, following manufacturer's protocol. DNA was extracted from 200 µL serum with 1 µL of the internal control (1X IPC DNA; Applied Biosystems, Foster City, CA, USA) and eluted in 50 µL buffer.

HBV quantification by Versant HBV DNA assay: The Versant HBV DNA Assay version 3.0 was performed in accordance with the manufacturer's protocol.

Preparation of standard: A sample with a very high viral load was selected and diluted in normal plasma to be used as a quantification standard. This diluted sample was ice-covered at -80 ºC in 200 µL aliquots in order to preserve its viral load. Then it was tested in triplicate and the obtained result was compared to the external reference

laboratory. This sample was extracted, diluted from 10^{-1} to 10^{-6} and used as a quantification control.

Statistical analysis: Statistical analysis was performed on the data obtained by in vivo studies. One-way analysis of variance (ANOVA) was accomplished of different variance. All through, the level of significance was chosen as less than 0.05 (i.e., $p < 0.05$).
