4.1 MATERIALS

4.1.1 Chemicals:

Table 4.1 List of chemicals used in the study

S. No.	Chemicals	Source
1	Dasatinib	MSN Laboratories, India
2	Gold chloride hydrate	Sigma-Aldrich, India
3	PLGA	Evonik Industries, India
4	Chitosan	Sigma-Aldrich, India
5	PEG Thiol	Sigma-Aldrich, India
6	PVP	Evonik Industries, India
7	Dimethyl sulfoxide	Sigma-Aldrich, India
8	Sodium carbonate	SD Fine Chemicals, India
9	Heparin sodium injection	Biological E. Limited, India
10	Calcium chloride	SD Fine Chemicals, India
11	Acetonitrile	Merck Ltd., India
12	Sodium carbonate	SD Fine Chemicals, India
13	Ethyl acetate	Hi Media, India
14	Diethyl ether	SD Fine Chemicals, India
15	Ethanol	Merck Ltd., India
16	Sodium hydroxide	SD Fine Chemicals, India
17	Acetone	Merck Ltd., India
18	Hydrochloric acid	Hi Media, India
19	Sodium chloride	SD Fine Chemicals, India
20	Fluorescein sodium	Hi Media, India
21	Methanol	SD Fine Chemicals, India
22	Calcium chloride	Merck Ltd., India
23	Potassium dihydrogen phosphate	SD Fine Chemicals, India
24	Membrane filters (0.22, 0.45 μm)	Hi Media, India
25	Potassium chloride	SD Fine Chemicals, India

4.1.2 Equipments

Table 4.2 List of major equipments used in the study

S. No.	Equipments	Source
1	Digital balance	Dewinter, India
2	Ultra probe sonicator	UP50H, Hielscher, USA
3	CO_2 incubator	Sanyo CO2 Incubator, Japan
4	Digital magnetic stirrer	IKA, Germany
5	UV-Visible spectrophotometer	UV-1800, Shimadzu, Japan
6	Vortex mixer	Fisher Scientific, India
7	pH meter	IKON Instruments, India
8	FTIR 8400S	Shimadzu, Japan
9	Stability chamber	NSW-175, India
10	Sonicator	Q 500, Q Sonica, USA
11	HPLC	Shimadzu, Japan & Agilent, USA
12	Zeta sizer	Beckman Coulter, USA
13	Electric oven	Cintex, India
14	Powder x-ray diffractometer	Rigaku, Japan
15	Micro pipettes	Thermo Scientific, USA
16	Cooling centrifuge	RC 4100F, Eltek, India
17	Lyophilizer	Labconco, USA
18	Particle size analyzer	Beckman Coulter, USA
19	FTIR spectrophotometer	FTIR-8400S, Shimadzu, Japan
20	Scanning electron microscope	Carl Zeiss, USA
21	Transmission electron microscopy	TECHNAI-20G^2, FEI, Czech Republic
22	Optical microscope	Dewinter Optical Inc., India
23	High resolution scanning electron microscopy	Nova NanoSem 450, FEI, Czech Republic
24	Energy dispersive x-ray spectoscopy	Ametek, USA
25	Atomic force microscopy	NT-MDT, Russia
26	Syringe filter	Millipore, USA
27	Dialysis membrane	12-14 kDa, Himedia, India
28	Confocal microscope	TCS SP2, Leica, Germany
29	Flow cytometer	BD FACSCalibur, Becton, Dickinson & Company, USA

4.1.3 Animals and cell lines

Table 4.3 List of animals and cell lines used in the study

S. No.	Animals and cell lines	Source
1	Dawley Sprague rats	Central Animal House, Banaras Hindu University, India
2	K562 (Human myeloid leukaemia cell lines)	ACTREC, Mumbai, India

4.1.4 Softwares

Table 4.4 List of softwares used in the study

S. No.	Software	Source
1	OriginPro 8	Origin Lab Corporation, MA, USA
2	Minitab 17	Minitab Inc., PA, USA
3	GraphPad Prism 8	GraphPad Software Inc., CA, USA
4	Kinetica 5.1	Thermo Scientific Inc., CA, USA

4.2 Preformulation studies

The preformulation studies are fundamental step in the development of a safe, stable and effective dosage form of a drug alone and in combination with the other excipients used in the formulation.

4.2.1 Physical observation

The identification of the drug was done by checking color, odour, and physical appearance. It was recorded and documented as evidence for comparing with the other batches during formulation.

Colour - White to off-white

Odour - Odourless

Physical appearance - Solid crystalline powder

4.2.2 FTIR interpretation

The pure drug of DSB was mixed with FTIR grade potassium bromide (KBr) in the proportion of (1:100), and made into small pellets by employing manual press. % transmittance (% T) of the pellets was recorded in diffuse reflectance mode in the spectral region of 4000–500 cm^{-1} operated at a resolution of 4 cm^{-1} by FTIR instrument. With the help of FTIR studies any physical and chemical interactions between various components used in the formulation can be investigated and it can be helpful in selection of excipients with suitable physicochemical compatibility.

4.2.3 Analytical method development for DSB by UV-Visible spectroscopy

4.2.3.1 Preparation of pH 7.4 PBS (phosphate buffered saline)

400 ml of distilled water was taken and to it 4.00 g sodium chloride, 0.72 g of disodium hydrogen phosphate, 0.12 g of potassium dihydrogen phosphate and 0.10 g of potassium chloride was added. The resultant solution was adjusted to the desired pH (typically pH ~ 7.4) and distilled water was added to a total volume of 500 ml [Sambrook et al., 1989].

4.2.3.2 Preparation of DSB stock solution

100 mg of DSB was weighed accurately and dissolved in 40 ml of pH 7.4 PBS in a volumetric flask (100 ml). To obtain a stock solution of drug concentration (1000 µg/ml), the volume was made up to the mark with pH 7.4 PBS. From the resultant solution, aliquots of 10 ml were pipetted out into 100 ml volumetric flasks and dilutions were made with pH 7.4 PBS to obtain concentration range of 30-100 µg/ml solutions. The resultant drug solutions were scanned for spectrum under 260 to 500 nm using Shimadzu UV-1800 UV-Visible Spectrophotometer [Sankar et al., 2009].

4.2.4 Analytical method development for biological samples (rat plasma) using HPLC and its validation

4.2.4.1 Chromatographic conditions

The HPLC (Shimadzu, Kyoto, Japan) system coupled with UV-visible detector (Shimadzu, SPD-20A) using C_{18} reverse phase column for the quantitative estimation of the drug. The mobile phase consisted of a mixture of pH 7.4 PBS:ACN (acetonitrile) in the ratio of 70:30 v/v, prepared freshly before the analysis. It was then filtered through 0.45 μm millipore filter unit, followed by degassing. The elution was performed at ambient room temperature with isocratic elution rate of 1 ml/min and volume of injection of 20 μl [Haouala et al., 2009]. The wavelength for detection was set at 325 nm according to the UV spectra of DSB.

4.2.4.2 Preparation of DSB standard solutions

A standard stock solution of DSB was prepared by dissolving 10 mg DSB in 10 ml of methanol. Standard working solutions of DSB (10-100 μg/ml) were obtained by serial dilution of the standard stock solution with pH 7.4 PBS and was stored in the refrigerator. It was found to be stable for at least two months, and no evidence of the analyte degradation was observed during this period of storage.

4.2.4.3 Preparation of standard plasma drug samples

Healthy Sprague Dawley rats of both sex having a body weight of 150-200 g were used for the animal studies. Central animal ethical committee of Institute of Medical Sciences, BHU, Varanasi approved (No. Dean/2017/CAEC/ 708) the experimental protocol. 200 μl of rat plasma sample was spiked with DSB to yield DSB concentrations ranging from 100-2000 ng/ml of DSB in 2 ml microcentrifuge tube and vortexed (IKA) for 5 min. The resultant drug solution in plasma was mixed with 400 μl of acetonitrile, vortexed for 2 min and further centrifuged for 10 min at 8000 rpm. The

supernatant sample was injected into HPLC system after filtering through 0.45 μm millipore filter unit.

4.2.4.4 Recovery

The recovery of DSB from the plasma was determined by comparing the peak area/absolute responses of extracted samples with the peak area/absolute responses of unextracted standard solutions containing the corresponding concentrations (100-2000 ng/ml) in the mobile phase. The quality control samples were prepared by spiking the blank plasma (sample without drug) with appropriate volume of DSB. Before proceeding to spiking, it was made sure by testing that the blank plasma had no endogenous interference at retention time of DSB.

4.2.4.5 Linearity, precision and accuracy

The linearity of the developed method was established by calibration curve of DSB in the plasma in the concentration range of 100-2000 ng/ml. The calibration curve of DSB was obtained by plotting the DSB concentrations versus the peak areas of DSB by regression analysis. Calibration curve of DSB was prepared by diluting standard solutions to get concentrations of 100, 200, 400, 600, 800, 1000 and 2000 ng/ml. The calibration curve requires r^2 (correlation coefficient) of 0.999 and each back calculated standard concentration should be ≤ 15% of the nominal value. Precision (intra-day and inter-day variation) was estimated by replicate analysis of seven spiked samples with seven different DSB concentrations (100-2000 ng/ml) within one day or seven successive days. The accuracy of the developed method was expressed as % bias and for intra-day and inter-day values to be acceptable, the value should be 100 ± 15 % and percentage relative standard deviation (% RSD) values should be < 15% over the calibration range. Precision and accuracy were established for DSB as per the US FDA guidance (bioanalytical method validation) [Kassem et al., 2013].

$$\% \text{ Bias} = \frac{\text{Observed concentration} - \text{Nominal concentration}}{\text{Nominal concentration}} \times 100$$

The least possible level of concentration resulting in a peak area of 3.3 times the baseline noise is known as limit of detection (LOD). The least possible level of concentration resulting in a peak area of 10 times the baseline noise is known as limit of quantitation (LOQ). From the signal-to-noise ratios, both LOD and LOQ were estimated and calculated by the following formulas:

$$\text{LOD} = \frac{3.3\ \text{SD}}{\text{m}} \times 100$$

$$\text{LOQ} = \frac{10\ \text{SD}}{\text{m}} \times 100$$

where SD = standard deviation of the y-intercept of the calibration curve and m = mean of the slope of the calibration curve.

4.3 Formulation development

The synthesis of GNPs by green reduction method using natural polymer (chitosan) and its subsequent capping utilizing limited amount of raw materials and time is the first step in the formulation development. Subsequently, the resultant GNPs were stabilized with polymer and loaded with the drug in order to preserve its stability and bioactivity during fabrication and release. The prepared nanoformulations were optimized by QbD approach and the optimized nanoformulations were studied extensively for solid state, physicochemical and morphological characteristics by various techniques. Further, they were evaluated for stability at different storage conditions, *in vitro* drug release and release kinetic studies, *in vitro* hemocompatibility study, cellular uptake study by confocal fluorescence microscopy, *in vitro* cytotoxicity study in K562 cell lines, cell apoptosis assay and *in vivo* pharmacokinetic study in Sprague Dawley rats in order to make them a promising system of DSB delivery in the treatment of CML.

4.3.1 Preparation of DSB loaded PVP stabilized chitosan capped GNPs (DSB-PVP-Ch-GNPs)

Chitosan (0.05 g) was added to 20 ml aqueous solution of 1.0 mM hydrogen tetrachloroaurate (III) hydrate and the solution was kept on the temperature controlled magnetic stirrer at 70^0C for 45 mins. The formation of Ch-GNPs was indicated by the development of a deep ruby red color of the resultant dispersion and confirmed with UV–Vis spectroscopy by recording a peak maximum at 525 nm, which is attributed to the SPR (surface plasmon resonance) band of GNPs. The synthesized Ch-GNPs were centrifuged at 12,000 rpm for 30 mins, washed and redispersed in deionized water to remove excess chitosan and gold. Finally, to remove any ionic impurities present in the Ch-GNPs dispersion, it was dialyzed using a dialysis tube for 12 hours.

For preparing DSB-PVP-Ch-GNPs, PVP (0.01 mg/mg of Ch-GNPs) and DSB (0.5 mg/mg of Ch-GNPs) in DMSO were added to Ch-GNPs dispersion and homogenized for 24 hours. Then, the resultant was kept for 12 hours at room temperature in order to allow proper interaction, sonicated for 30 minutes and subjected to stirring for 4 hours. The drug loaded polymeric gold nanocarriers were centrifuged for 15 mins at 10,000 rpm and was dialyzed by using deionized water for 12 hours. The resultant dispersion was freeze dried and the lyophilized nanoformulation was stored in a desiccator until further use (Figure 4.1).

Figure 4.1 Preparation of DSB loaded PVP stabilized chitosan capped gold nanoparticles

4.3.1.1 Factorial design for formulation development

Initially, preliminary screening experiments were performed by design of experiment (DOE) approach in order to establish the most important factors and the proper ranges in which the optimal range lie, followed by RSM (response surface methodology). Plackett-Burman design (PBD) is the most suitable method for screening the main factors and reducing the number of experiments. It was also used to assess the correlations between the most important factors and the responses. The high and low levels of each factor and their response were evaluated by preliminary screening experiments. Pareto chart was employed in PBD in order to select the most important factors that are responsible for the significant effects.

4.3.1.2 Graphical drawing, optimization and statistical analysis

Polymer concentration (PC), stirring time (ST) and sonication time (SoT) were identified as the most significant variables through PBD and they were tested on the responses viz. particle size (PS), entrapment efficiency (% EE) and zeta potential (ZP). Box-Behnken design (BBD) (3-factor, 3-level) was used on the basis of the preliminary trials to study the effect of each factor on the responses [Singh et al., 2005a, Singh et al.,

2005b]. BBD was selected for the present study as it generates fewer runs with 3 factors and also it is suitable for deriving polynomial equation and exploring quadratic response surfaces. BBD consisted of 15 runs, which were done in triplicate. Analysis of variation (ANOVA) helps to recognize the most significant factors that influence the responses and also determines the fitness of the model. It was employed in order to establish the magnitude and significance of the effects of the main factors and their interactions by applying p-value (probability value). The fitness of the model was examined by coefficient of determination (R-sq) and signal to noise (F-value). Minitab 17 software was used to analyze the response surface of the factors and responses inside the experimental domain. Polynomial equation derived by BBD is as follows

$$Y_i = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_{11} X_1^2 + \alpha_{22} X_2^2 + \alpha_{33} X_3^2 + \alpha_{12} X_1 X_2 + \alpha_{13} X_1 X_3 + \alpha_{23} X_2 X_3$$

where, Y_i = measured response, α_0 = intercept, α_1 to α_{33} = regression coefficients of Y_i. X_1, X_2 and X_3 = factors.

4.3.1.3 UV-Vis absorption spectrophotometry

UV-Vis absorption spectrophotometry was used to monitor the formation and stability of the GNPs. It can be used as a valuable tool for the detection of the GNPs in a fast and real time manner, and also provides information about its concentration and aggregation. The absorption spectra of the GNPs were procured in the wavelength range of 300 - 800 nm.

4.3.1.4 Determination of PS and ZP

The PS and size distribution of the DSB-PVP-Ch-GNPs were determined by DLS (dynamic light scattering) using Desla Nano Common (Beckman Coulter, UK) instrument. It is used to accurately measure the hydrodynamic size of the nanoparticles

in the solution. In this technique, the DSB-PVP-Ch-GNPs solution was illuminated and its scattering intensity was recorded at a fixed scattering angle with a photon detector. The samples were diluted with ultrapure milli-Q water to obtain a concentration close to 100 μg/ml, and filtered through 0.45 μm membrane syringe filter before loading the samples into the cuvette in order to get rid of the large interfering particulate matter. The ZP of the DSB-PVP-Ch-GNPs was determined by laser doppler velocimetry (LDV) using Desla Nano Common (Beckman Coulter, UK) instrument, in which an electrical field is applied across the DSB-PVP-Ch-GNPs sample and the electrophoretic mobility of nanoparticles was measured. Nanoparticles having ZP in the range of −10 to +10 mV are considered as neutral, while less than +30 mV or more than -30 mV are considered as strongly cationic or anionic, respectively [Clogston and Patri, 2011].

4.3.1.5 Determination of % EE

The % EE of DSB-PVP-Ch-GNPs was determined by centrifugation method by separating the supernatant free drug content. The nanoparticles were centrifuged for 30 mins at 12,000 rpm in order to separate the unentrapped drug from the nanoparticles. After centrifugation, the supernatant containing free drug was separated and pH 7.4 PBS was added and vortexed for 5 mins. The resultant solution was subjected to filtration through 0.22 μm filter and the filtrate was analyzed using UV–Vis absorption spectrophotometer [Govender et al., 1999]. The % EE was calculated by using the formula:

$$\% \text{ EE} = \frac{\text{Total amount of drug added} - \text{Amount of free drug in the supernatant}}{\text{Total amount of drug added}} \times 100$$

4.3.1.6 FTIR and XRD studies

FTIR (Fourier transform infrared spectroscopy) was employed to examine the physical and chemical interaction between the drug and excipients before and after the formulation. FTIR spectra of DSB, PVP, Ch, Ch-GNPs and DSB-PVP-Ch-GNPs were obtained by using FTIR spectrophotometer. Detail method is given in sub-section 4.2.2

XRD (x-ray diffraction) was employed to measure the physical properties of DSB, PVP, Ch, Ch-GNPs in pure form and inside the DSB-PVP-Ch-GNPs. XRD measurements were carried out to check the crystallinity as well as any change in the physical state of the drug. The study was performed on x-ray diffractometer (Rigaku, Japan) in which x-ray radiation source was Ni-filtered Cu Kα 1, the amperage was 15 mA and tube voltage was 40 kV. The XRD diffractograms were recorded at a step width of 0.02^0, scan speed of 10^0 min^{-1} and 2θ scanning range of 20^0 - 80^0.

4.3.1.7 TEM

TEM (transmission electron microscopy) was used to investigate size, dispersity and shape of DSB-PVP-Ch-GNPs. The sample was prepared by placing a very small drop of the DSB-PVP-Ch-GNPs dispersion onto a carbon coated copper grid and was allowed to dry at the room temperature. TEM micrographs of DSB-PVP-Ch-GNPs were obtained using an FEI Tecnai G^2 20 X-TWIN (FEI, Czech Republic) instrument.

4.3.1.8 *In vitro* drug release study

In vitro drug release profile of DSB from DSB-PVP-Ch-GNPs was determined using modified dialysis bag technique [Soni et al., 2016]. DSB-PVP-Ch-GNPs dispersion was dialyzed against 100 ml pH 7.4 PBS and placed in a dialysis bag (mol. wt. cut off between 12-14 kDa) on a temperature controlled magnetic stirrer at 37 ± 0.5^0 C with 100 rpm of stirring. At fixed intervals of time, the whole volume of receptor solutions were replaced with the same volume of fresh pH 7.4 PBS in order to maintain

the sink conditions and the solution containing released DSB was quantitatively analyzed on UV-visible spectrophotometer at 325 nm λ_{max}.

4.3.2 Preparation of DSB loaded PEG stabilized chitosan capped GNPs (DSB-PEG-Ch-GNPs)

The detail method of preparation of chitosan capped GNPs is given in sub-section 4.3.1.

For preparing DSB-PEG-Ch-GNPs, PEG (0.01 mg/mg of Ch-GNPs) was added to Ch-GNPs dispersion and continuously stirred for 24 hours. Then, DSB (0.5 mg/mg of Ch-GNPs) in DMSO was added to the Ch-GNPs dispersion, kept at room temperature for 12 hours for their proper interaction and further stirred continuous for another 6 hours. The drug loaded polymeric gold nanocarriers were sonicated and centrifuged for 30 mins at 12,000 rpm. The resulting dispersion was dialyzed against deionized water for 12 hours and subjected to freeze drying. The lyophilized nanoformulation was stored at ambient storage conditions for further use.

4.3.2.1 Risk assessment studies

Risk is a combination of the likelihood of occurrence of a damage and the severity of that damage. Each and every component that is used in the development of the pharmaceutical product exhibits some risk. To reduce the risk and avoid the potential product failure, risk assessment is performed to identify the critical quality attributes (CQAs) that may affect the product's final quality. It is also useful to raise the quality of process or method and it is determinant for effect of input variable on process or method. Failure modes and effects analysis (FMEA) is an important tool to identify all the important potential risks based on rank modes of relative usefulness to prioritize the CQAs as low, medium and high [Sangshetti et al., 2014]. Table 4.5 shows

the prioritization levels of the factors ahead of employing design of experiments to optimize the manufacturing process of GNPs.

Table 4.5 Prioritization of critical quality attributes based on failure modes and effects analysis

CQAs (Factors)	Responses		
	Particle size	Zeta potential	% Entrapment efficiency
Polymer type	Low	Low	Low
Polymer concentation	High	Low	High
Stirring speed	Medium	Low	Low
Stirring time	High	Medium	High
Solvent type	Low	Low	Low
Solvent ratio	Medium	Low	Medium
Sonication time	High	Medium	High
Sonication frequency	Low	Low	Low
Temperature	Low	Low	Low
Centrifugation time	Low	Low	Medium
Centrifugation speed	Low	Low	Medium

4.3.2.2 Factorial design

To establish the significant factors and their proper optimal range, DOE approach was used for the preliminary screening experiments followed by RSM. PBD is the best suitable method for screening the significant factors thereby reducing the number of experimental runs [Cavazzuti, 2013]. PBD was employed by using Minitab

17 software in order to evaluate the interaction between the significant factors and responses. The preliminary screening experiments with each factor (A - polymer concentration, B - stirring speed, C - stirring time, D - sonication time, E - sonication frequency, F - temperature, G - centrifugation time, H - centrifugation speed) at low and high levels and the responses (Y_1 - particle size, Y_2 - % entrapment efficiency and Y_3 - zeta potential) were evaluated. Pareto chart was employed in PBD in order to determine the main factors that were responsible for the significant effects.

4.3.2.3 Graphical drawing, optimization and statistical analysis

PC, ST and SoT were recognized as the significant factors through preliminary screening by PBD. BBD was used to study the effect of each factor on response based on the preliminary screening [Singh et al., 2005a, Singh et al., 2005b]. 15 experimental runs were generated and all the experiments were performed in triplicate. ANOVA is an important tool employed to identify the fitness of the model and also the significant factors that affect the responses by coefficient of determination (R-sq), signal to noise (F-value) and probability value (p-value). The response surface of factors and responses of the experimental domain was analyzed by Minitab 17 software. Polynomial equation derived by BBD is as follows

$$Y_i = X_0 + X_1 A + X_2 B + X_3 C + X_{11} A^2 + X_{22} B^2 + X_{33} C^2 + X_{12} AB + X_{13} AC + X_{23} BC$$

where, Y_i = measured response, X_0 = intercept, X_1 to X_{33} = regression coefficients of Y_i. A, B and C = factors.

4.3.2.4 Physical characterization of DSB-PEG-Ch-GNPs

UV-Vis absorption spectrophotometry was used to monitor the formation and stability of the GNPs. It can be used as a valuable tool for the detection of the GNPs in a fast and real time manner, and also provides information about its concentration and

aggregation. The absorption spectra of the GNPs were procured in the wavelength range of 300 - 800 nm.

The PS and size distribution of the DSB-PEG-Ch-GNPs were determined by DLS. The samples were diluted with ultrapure milli-Q water to obtain a concentration close to 100 μg/ml, and filtered through 0.45 μm membrane syringe filter before loading the samples into the cuvette in order to get rid of the large interfering particulate matter. The ZP of the DSB-PEG-Ch-GNPs was determined by LDV in which an electrical field is applied across the DSB-PEG-Ch-GNPs sample and the electrophoretic mobility of nanoparticles was measured. The PS and ZP measurements were performed by using Desla Nano Common instrument.

The % EE of DSB-PEG-Ch-GNPs was determined by centrifugation method by separating the supernatant free drug content. The nanoparticles were centrifuged for 30 mins at 12,000 rpm in order to separate the unentrapped drug from the nanoparticles. After centrifugation, the supernatant containing free drug was separated and pH 7.4 PBS was added and vortexed for 5 mins. The resultant solution was subjected to filtration through 0.22 μm filter and the filtrate was analyzed using UV–Vis absorption spectrophotometer [Govender et al., 1999]. The % EE was calculated by using the formula:

$$\% \text{ EE} = \frac{\text{Total amount of drug added} - \text{Amount of free drug in the supernatant}}{\text{Total amount of drug added}} \times 100$$

4.3.2.5 Solid state characterization

FTIR (Fourier transform infrared spectroscopy) was employed to examine the physical and chemical interaction between the drug and excipients before and after the formulation. FTIR spectra of DSB, PEG, Ch, Ch-GNPs and DSB-PEG-Ch-GNPs were obtained by using FTIR spectrophotometer. For that, all the samples were individually

mixed with KBr, compressed into a very thin disc in a manual press and the % transmittance (% T) of the pellets was recorded in diffuse reflectance mode in the spectral region of 4000–500 cm^{-1} operated at a resolution of 4 cm^{-1} by FTIR instrument..

XRD (x-ray diffraction) was employed to measure the physical properties of DSB, PEG, Ch, Ch-GNPs in pure form and inside the DSB-PEG-Ch-GNPs. The study was performed on x-ray diffractometer in which x-ray radiation source was Ni-filtered Cu Kα 1, the amperage was 15 mA and tube voltage was 40 kV. The XRD diffractograms were recorded at a step width of 0.02^0, scan speed of 10^0 min^{-1} and 2θ scanning range of 20^0 - 80^0.

4.3.2.6 Physicochemical and morphological characterization

High resolution scanning electron microscopy (HR-SEM) was used to confirm the production and examine the morphology of DSB-PEG-Ch-GNPs. FEI Nova NanoSem 450 (FEI, Czech Republic) instrument was used to acquire HR-SEM micrographs. The sample was prepared by placing a very small drop of the DSB-PEG-Ch-GNPs dispersion onto a carbon coated copper grid. It was allowed to dry at the room temperature and coating with gold palladium particles to make it conductive by using high vacuum evaporator (Quorum Q150R ES). HR-SEM micrographs were taken at suitable magnifications.

Energy dispersive x-ray spectroscopy (EDXS) analysis was done to determine the elemental composition of the DSB-PEG-Ch-GNPs, EDXS spectrum was acquired using EDAX (Ametek, NJ, USA) instrument attached with the HR-SEM. The EDXS spectrum was recorded by focusing on the densely occupied DSB-PEG-Ch-GNPs region and acquired the electron micrographs in the spot profile mode.

TEM was employed to investigate shape, size and dispersity of the DSB-PEG-Ch-GNPs. The sample was prepared by placing a very small drop of the DSB-PEG-Ch-

GNPs dispersion onto a carbon coated copper grid and was allowed to dry at the room temperature. TEM micrographs of DSB-PEG-Ch-GNPs were obtained using an FEI Tecnai G^2 20 X-TWIN instrument. In conjunction with TEM, selected area electron diffraction (SAED) study was performed to assess the amorphous nature of the DSB-PEG-Ch-GNPs.

Atomic force microscopy (AFM) was employed to examine the surface morphology of DSB-PEG-Ch-GNPs. The sample was prepared by placing a very small drop of the DSB-PEG-Ch-GNPs dispersion onto mica surface and was allowed to dry at the room temperature in order to form a thin film. To visualize the 3D and 2D images, atomic force microscope (NT-MDT, NTEGRA Probe Nanolaboratory, Russia) was used.

4.3.2.7 Stability study

DSB-PEG-Ch-GNPs were subjected to accelerated (40 ± 2^0 C / 75 ± 5% RH), room temperature (25 ± 2^0 C) and refrigerated (4 ± 1^0 C) conditions to evaluate the effect of stress conditions as per ICH (International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) guidelines. Freshly prepared DSB-PEG-Ch-GNPs were lyophilized, sealed in glass vials and placed in three different storage conditions. The samples were withdrawn at different time intervals (0, 2, 4 and 6 months) and analyzed for PS, % EE and ZP.

4.3.2.8 *In vitro* drug release studies

In vitro drug release profile of DSB from DSB-PEG-Ch-GNPs was determined using modified dialysis bag technique [Soni et al., 2016]. DSB-PEG-Ch-GNPs dispersion was dialyzed against 100 ml pH 7.4 PBS and placed in a dialysis bag (mol. wt. cut off between 12-14 kDa) on a temperature controlled magnetic stirrer at 37 ± 0.5^0 C with 100 rpm of stirring. At fixed intervals of time, the whole volume of receptor

solutions were replaced with the same volume of fresh pH 7.4 PBS in order to maintain the sink conditions and the solution containing released DSB was quantitatively analyzed on UV-visible spectrophotometer at 325 nm λ_{max}.

To evaluate the mechanism of DSB release, the *in vitro* DSB release data from the optimized nanoformulation was fitted to five primarily applied mathematical models [Peppas, 1985] such as zero order (cumulative % DSB released vs. time), first order (log cumulative % DSB remaining to be released vs. time), Higuchi square root model (cumulative % DSB released vs. square root of time), Hixon-Crowell cube root model (cube root of DSB % remaining to be released vs. cube root of time) and Korsmeyer-Peppas model (fraction of DSB released vs. time).

Zero order model	:	$Q_t = Q_0 + C_0t$
First-order model	:	$Q_t = In\ Q_0 + C_1t$
Higuchi square root model	:	$Q_t = Q_0 + C_Ht^{1/2}$
Korsmeyer-Peppas model	:	$Q_t/Q_\infty = Q_0 + C_Pt^n$
Hixon-Crowell model	:	$Q_t^{1/3} = Q_0^{1/3} - C_{HC}t$

where Q_t = amount of DSB released at time t, Q_0 = initial amount of DSB released, Q_t/Q_∞ = fraction of DSB released at time t, C_0 = zero order release constant, C_1 = first order release constant, C_H = Higuchi release constant, C_{HC} = Hixson-Crowell release constant, C_p = Korsmeyer-Peppas release constant and n = release exponent.

4.3.3 Preparation of DSB loaded PLGA stabilized chitosan capped GNPs (DSB-PLGA-Ch-GNPs)

GNPs were synthesized by natural reduction method in which hydrogen tetrachloroaurate(III) hydrate was reduced by chitosan. In this process chitosan (0.05 g) was added to 20 ml gold chloride hydrate solution and was stirred magnetically at 70 $^{\circ}$C for 45 minutes until the solution changes to deep ruby red colour. The synthesized

GNPs were allowed to cool at room temperature. The formation of Ch-GNPs was indicated by the development of a deep ruby red color of the resultant dispersion and confirmed with UV–Vis spectroscopy by recording a peak maximum at 525 nm, which is attributed to the SPR (surface plasmon resonance) band of GNPs. The resultant dispersion was further washed to remove excess chitosan and gold, and finally redispersed in deionized water.

For preparing DSB loaded GNPs, PLGA (0.01 mg/mg of Ch-GNPs) was added to Ch-GNPs solution and stirred for 24 hours. Then DSB (0.5 mg/mg of Ch-GNPs) in DMSO were added while homogenizing (Ika T25 Ultra Turrax, Germany) the GNPs solution for 4 hour, followed by sonication for 15 minutes (Q Sonica Q 500, Newtown, USA). To allow proper interaction, the resulting solution was stored at room temperature for 12 hours followed by stirring for 6 hours. Further, the resulting solution was subjected to centrifugation (Remi, Vasai, India) for 30 minutes at 10,000 rpm followed by freeze drying (Labconco, Kansas, USA) to obtain lyophilized gold nanoformulation of DSB (Figure 4.2).

Figure 4.2 Preparation of DSB loaded PLGA stabilized chitosan capped gold nanoparticles

4.3.3.1 Cause-effect relationship: Ishikawa fish bone

To identify the probability of potential product failure due to failure(s) or risk(s), a risk assessment was performed. Ishikawa fish bone diagram was employed to configure the risk assessment and to determine the cause-effect relationship between the factors and the critical quality attributes (CQAs) [Beg et al., 2015]. The PEMMEM (personnel, equipment, materials, method, environment, and measurement) most likely affecting the CQAs were enlisted in Ishikawa fish bone diagram (Figure 4.3). By prior scientific knowledge PS, % EE and ZP were identified as the key CQAs in the present study [Yerlikaya et al., 2016, Rahman et al., 2010].

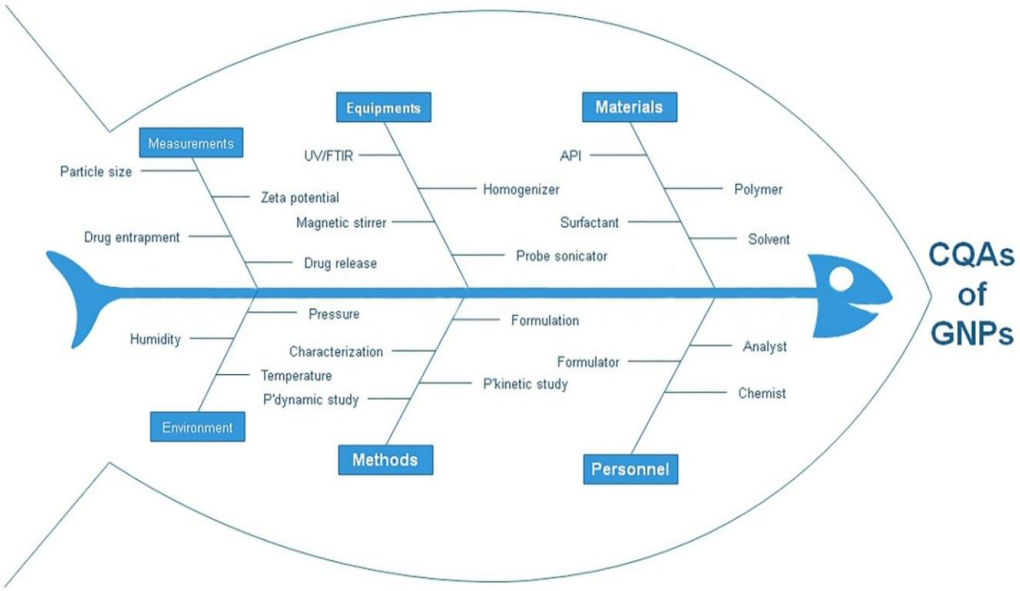

Figure 4.3 Ishikawa fish bone diagram showing the cause-effect relationship between variables for the critical quality attributes of DSB-PLGA-Ch-GNPs

4.3.3.2 Risk assessment screening: PBD

PBD was employed to screen and evaluate the significant risk factors that may influence the identified CQAs. The preliminary screening experiments with factors and the CQAs were evaluated and coded as "+" and "-" for high levels and low levels respectively for simplification. PBD was constructed with eight factors, three responses and twelve experiments using Minitab 17 software (Minitab Inc., Pennsylvania, USA), and statistical analysis was performed. ANOVA was employed to test the significance of PBD and coefficients for each factor [Vardhan et al., 2017]. Further, Pareto chart was employed to choose the main factors that have a significant effect on CQAs (responses).

4.3.3.3 Optimization of nanoformulation: 2^3 Full factorial design

The effects of the three main factors (PLGA concentration (PC), sonication time (SoT) and stirring time (ST)) were tested on the responses (PS, ZP, and % EE). By the preliminary screening, 23 Full factorial design (FFD) (3-factor, 2-level) was applied to optimize the DSB-GNPs. FFD was selected for the present study as it generates fewer

experimental runs with three factors and also it is appropriate for exploring the quadratic equation, deriving the polynomial equation and plotting response surface plots. The experimental design was constructed and evaluated by using Minitab 17 software. Eight batches were prepared as per FFD and ANOVA was employed to determine the significance of the experimental model. The polynomial equation generated by the FFD elucidating the effect of factors on each of the responses is as follows:

$$Y_i = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_{12} X_1X_2 + \alpha_{13} X_1X_3 + \alpha_{23} X_2X_3 + \alpha_{123} X_1X_2X_3$$

Where Y_i is the response, α_0 is the intercept, α_1 to α_{123} is the associated coefficients coded values of the responses. X_1, X_2, and $X_3$are the coded value of the factors.

4.3.3.4 Physical characterization of DSB-PLGA-Ch-GNPs

The detail method of formation and stability of the GNPs, PS, size distribution, ZP and % EE is given in sub-section 4.3.2.4.

4.3.3.5 Solid state characterization

FTIR is primarily used to identify the presence of certain functional groups in the molecule. When infra red radiation is passed through the molecule of interest, it absorbs certain frequency of radiation that is characteristic to its structure and provides the molecular fingerprint of the molecule. FTIR is widely used for analyzing the composition of the sample mainly because it is very sensitive and rapid technique with better signal to noise ratio. FTIR result in a qualitative analysis of different kind of materials as different materials produce different FTIR spectrum. FTIR spectra of optimized nanoformulation, DSB, PLGA, Ch, and GNPs were obtained in order to examine any possible interactions between the drug-excipients before and after the formulation of DSB-PLGA-Ch-GNPs. The spectra were obtained by using FTIR

spectrophotometer. The % transmittance was recorded in the spectral region of 4000-500 cm^{-1}.

XRD is a very important, powerful, highly qualitative and non destructive technique that is used to determine primarily the characteristic and structural details of nanoformulation. It was used to examine the physical properties of drug and excipients in pure form and in the formulation, to check the crystallinity as well as any change in the drug's physical state after the entrapment. The XRD spectra of optimized nanoformulation, DSB, PLGA, Ch, and GNPs were obtained by using x-ray diffractometer. The diffractograms were recorded by using 15 mA amperage, 40 kV tube voltage, 10° min^{-1} scan speed, 0.02° step width and 20° to 80° 2θ scanning range.

4.3.3.6 Physicochemical and morphological characterization

The detail method of HR-SEM, EDXS, TEM, SAED and AFM is given in sub-section 4.3.2.6

4.3.3.7 Stability study

The detail method of stability study is given in sub-section 4.3.2.7

4.3.3.8 *In vitro* drug release study

The detail method of *in vitro* drug release study is given in sub-section 4.3.2.8

4.3.3.9 *In vitro* hemocompatibility study

It was performed in order to evaluate the hemolysis. This study is necessary for any dosage form that is intended to administer intravenously to show its hemocompatibility. Through retro orbital plexus of rat, the blood sample was collected from and centrifuged for 10 mins at 4000 rpm. To the separated erythrocyte sample equal volume of PBS solution was added and again centrifuged for 10 mins at 4000 rpm. Different concentrations of optimized nanoformulation (10-200 μg/ml) were added to 2 ml of erythrocyte suspension and incubated at 37^{o} C for 45 mins [Fornaguera et al.,

2015]. The resulting sample was stained with hematoxylin and eosin (H&E) stain, dried on the glass slide and enclosed with cover slip. Dewinter optical microscope was used to visualize the resulting blood smear.

4.3.3.10 Cellular uptake study by confocal fluorescence microscopy

K562 human myeloid leukaemia cell lines were cultured, seeded and incubated in multi well culture plates. Optimized nanoformulation of GNPs (2 µg/ml) was injected into the cell line suspension. Meanwhile, the K562 cell line (ACTREC, Mumbai, India) suspension with DSB was taken and both were incubated at 37 ^{0}C in a 5 % CO_2 incubator for 4-6 hours. K562 cells were covered with coverslips, fixed with an aqueous solution of paraformaldehyde (4 % v/v) and washed thrice with PBS solution [Panyam et al., 2003]. Laser scanning spectral confocal microscope (TCS SP2, Leica, Wetzlar, Germany) was used to generate the images.

4.3.3.11 *In vitro* cytotoxicity assay

It was performed by SRB (sulforhodamine B) assay [Skehan et al., 1990]. To determine the cell density on the basis of cell protein content measurement and thereby investigating the cell based cytotoxicity [Vichai and Kirtikara, 2006], human myeloid leukaemia cell lines (K562) were maintained in 96 well plates containing RPMI (Roswell Park Memorial Institute) - 1640 medium and incubated for 24 hours (37 $^{\circ}$C, 100 % RH, 95 % air and 5 % CO_2). After the stipulated time, the well plates containing cells were treated with different concentrations (10, 20, 40 and 80 µg/ml) of experimental drugs (DSB (pure drug), Adriamycin® (standard) and optimized nanoformulation) and incubated for 48 hours. Cold trichloro acetic acid was added to the well plates followed by incubation for 1 hour at 4 $^{\circ}$C in order to fix them in situ. Finally, to stain the cells SRB solution was added to each of the well plates and incubated for 20 minutes at room temperature. After staining, the unbound dye was

removed, and the bound stain was then eluted with the help of trizma base and the absorbance was recorded at 540 nm wavelength [Reddy et al., 2019]. Percentage cell growth and percentage growth inhibition were calculated for test wells relative to control wells.

4.3.3.12 Cell apoptosis assay

For cell apoptosis assay, K562 cells were cultured and at a density of 1 x 10^5 cells/well, the cells were seeded into 24-multiwell plates containing 10% fetal bovine serum (FBS) in RPMI medium. K562 cells were treated with different concentrations of optimized nanoformulation of GNPs (0, 5, 10 and 15 µg/ml) and incubated for 6, 12 and 24 hours in an incubator contained with 5% CO_2 at 37 ^{0}C. Then K562 cells were collected, washed with ice-cold PBS and re-suspended in binding buffer (1 µg/ml propidium iodide (PI) and 1 µg/ml of annexin V- fluorescein isothiocyanate (FITC)). After that, cell specimens were incubated for 15 min at room temperature in a light protected area and analyzed by using BD FACSCalibur™ flow cytometry system (Becton, Dickinson and Company, New Jersey, USA).

4.3.3.13 *In vivo* pharmacokinetic study

Healthy Sprague Dawley rats of both sex having a body weight of 150-200 grams were used for the *in vivo* pharmacokinetic study. Central animal ethical committee of Institute of Medical Sciences, BHU, Varanasi approved (No. Dean/2017/CAEC/708) the experimental protocol. The rats were randomly divided into 3 groups (pure DSB, optimized nanoformulation, and control) containing 6 rats in each group. The rats were housed in polypropylene cages at standard animal house conditions and were fasted overnight with water ad libitum. Pure DSB (32.8 mg/kg) and optimized nanoformulation (equivalent to 32.8 mg/kg of pure DSB) were dissolved in propylene glycol/water (50:50) mixture and administered by intravenous route through

the tail vein. The blood samples from each group were collected under ether anesthesia at various time points through the retro orbital plexus and centrifuged for 10 mins at 4000 rpm. The separated plasma samples were deproteinized with acetonitrile, and the supernatant was stored at -80° C until analysis by HPLC. 20 µl of plasma samples were injected into C_{18} reverse phase column maintained at isocratic elution rate (1 ml/min). The mobile phase consists of pH 7.4 PBS and ACN (70:30). Peak area versus time of blood collection data was recorded. Drug concentrations in each collected blood samples were estimated from calibration curve and plots were made between plasma drug concentrations versus time. Kinetica 5.1 software was used for the determination of pharmacokinetic paramaters which was done by the non-compartmental method [Vijayakumar et al., 2016].

4.3.3.14 Statistical analysis

All the experimental data was expressed as mean ± SEM (standard error of the mean). Statistical analysis of the data was performed by ANOVA followed by Bonferroni post test and Student-Newman-Keuls multiple comparisons tests wherever necessary. OriginPro 8 software (Origin Lab Corporation, MA, USA) was used for graph representation. GraphPad Prism 5 (GraphPad Software Inc., CA, USA) software was used for statistical analysis. A *p*-value < 0.05 was considered as statistically significant. In the case of formulation optimization, Minitab 17 (USA) was used for primary factor screening and response surface methodology. Kinetica 5.1 (Thermo Scientific Inc., CA, USA) was used for pharmacokinetics study.