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Development of Nitazoxanide Loaded Polymeric Nanocarriers: Box Behnken Experimental Design Based Optimisation and Characterisation

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Authors' contributions

This work was carried out in collaboration among all authors. Author CB designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SS and SK managed the analyses of the study. Author SAR managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The current investigation is focused on formulation, optimisation and characterisation of polymeric based nanomaterial. Nitazoxanide (NTZ) loaded polymeric nanoparticles were prepared by homogenisation technique using Eudragit RL100 as a polymer matrix and Poly vinyl alcohol (PVA) as a cross linking agent. NTZ was used as a model drug and investigated for preformulation parameters along with excipients, identification of concentration for optimization, selection of independent (X) and dependent (Y) variables and characterisation of optimised formulation. Polymeric nanoparticles were obtained after optimization using 3^3 factorial design by Box Behnken Design expert (BBD). The role and influence of key process variables i.e. concentration of polymer, concentration of cross linking agent and speed of rotation of homogeniser at their respective three different levels for the optimisation of formulation were also investigated. The synthesised optimised polymeric nanoparticles were further characterised by dynamic light scattering (DLS) for its particle size (137.11nm), PDI (0.180) and zeta potential (33.4 mV) while X-ray diffraction (XRD)

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was used to justify the amorphous and crystalline nature of drug and excipients. Transmission electron microscopy (TEM) further revealed surface geometry of these nanoparticles being spherical in shape, drug entrapment efficiency (%DEE) was found to be 81.89% and *in vitro* release studies showed sustained drug release effect. The antimicrobial activity against *Pseudomonas aeruginosa, Streptococcus mutans and Escherichia coli* was also determined.

Keywords: Polymeric nanoparticles; Box Behnken Design expert; nitazoxanide; dynamic light scattering; antimicrobial activity.

1. INTRODUCTION

As per the estimated data given by WHO for parasitic infections, there are 48.4 million cases and 59,724 deaths annually announced in 8.78 million (Disability Adjusted Life Years) DALYs. This estimated calculated data signifies an imperative step onward in consideration the influence of parasitic diseases worldwide and locally. The disease load from parasites is extremely central and results in substantial morbidity and mortality between susceptible populations, [1] therefore we have to focus on the novel formulation development specially nanomaterials using parasitic agent as a model drug. So these type of alteration in formulation of the material in nanotechnology often yield a product with improved prospective as well as inspire the researchers through the diverse disciplines to further exploration of nanomaterials in different pharmaceutical field. Polymeric nanoparticles are fundamental component of nanomaterial and physically they are colloidal particles having dimension range from 10-1000 nm in size and are accumulated from a diversity of non-biodegradable and biodegradable polymers. The polymers containing nanoparticles have been demonstrated for various applications like imaging, drug delivery, detection of diseases and various other pharmaceutical applications [2,3]. The drug is dissolved, entrapped, captured in a polymeric matrix in a nanosized range material. However, polymeric containing nanoparticles improve drug therapeutic efficacy, site specific targeting effect and decreased toxicity as well as occurrence of adverse drug effect [4]. The versatile features of polymeric nanoparticles such as defence of active molecules from deprivation, enhancement in pervasion of the active molecules across gastro intestinal tract, prolonged release of the entrapped drug, decrease in dosing incidence, increase solubility in water which astounded the physiological barriers to deliver the drugs at the target sites etc [5]. Therefore, the selection of polymers for encapsulation does depends upon the various factors for design of nanomaterials and their biocompatibility [6]. This investigation utilized a cationic polymer Eudragit RL100 which is basically a copolymer of methyl methacrylate, ethyl acrylate and a less concentration of a methacrylic acid ester with 4° ammonium groups. The ammonium groups are present as salts and make the polymers permeable [7]. It is less soluble in water but swells in digestive fluids and independent at the physiological pH but the cationic charge facilitates rapid permeation through the intestinal mucosa [5]. The drug payload can be transported through the process of diffusion. Eudragit RL 100 polymer is also used as a film former agent for solid dosage form and for the preparations of time-controlled drug delivery formulations [8]. Polymeric nanoparticles utilized Eudragit RL100 as a polymer and polyvinyl alcohol (PVA) as cross linking agent using NTZ as a model drug. Chemically, NTZ is 2-(acetyloxy)-*N*-(5-nitro-2thiazolyl) benzamide. The drug molecule is quickly converted into its active metabolite (desacetyl derivative tizoxanide). The chemical formula of NTZ is $C_{12}H_9N_3O_5S$, its molecular weight is 307.3 Da. [9]. It is an antiprotozoal agent used against wide range of infections such as *Cryptosporidia*, Hepatitis C and also shown a wide spectrum of pharmacological action in infectious and neoplastic diseases. Around 2/3rd and 1/3rd NTZ oral dose is excreted in faeces and in urine respectively [10]. The pharmacological activity of NTZ is supposed due to interference with pyruvate-ferrodoxin oxidoreductase (PFOR), enzyme dependent electron transfer reaction which is essential to anaerobiotic energy breakdown.

In any practical investigation, it is significant to learn the procedure engineering involved in formulation development to attain reliable product attributes [11]. Therefore, experimental design could be an appropriate step for the optimisation of drug loaded in nanoparticles by taking various independent and dependent variables under consideration. Experimental design is therefore a legalized and beneficial tool for the formulation and optimization of

experimental events with a minor amount of observations whereas still providing the preferred information on the relationship among the experimental and the response variables [12]. The design expert Box–Behnken experimental design (BBD) is a category of rotatable IInd order proposals based on 3 level incomplete factorial design which is proficiently predict non-linear models as associated to 2 level designs. Experimental designing is also predict the optimal results in the form of mathematical and graphical representation [13].

Thus, this investigation was envisioned to design, optimize and evaluate polymeric nanoparticles with a model drug NTZ, using 3^3 factorial design for the optimisation of nanoformulation. The effect of three experimental variables on the evaluation of nanoparticles were studied in terms of their particle size, polydispersive index (PDI) and zeta potential. This work included the effect of NTZ loaded polymeric nanoparticles on various microbes like *Pseudomonas aeruginosa, Streptococcus mutans and Escherichia coli*.

2. MATERIALS AND METHODOLOGY

2.1 Materials

NTZ was received as a gift sample from Ind-Swift Labs. Ltd., Jammu & Kashmir, India. Eudragit RL 100, Polyvinyl alcohol (PVA) and acetone were purchased from Sigma-Aldrich, New Delhi, India. All other chemicals used were of analytical grade and purchased from Sigma-Aldrich, New Delhi, India.

2.2 Methodology

2.2.1 Preliminary formulation study

Preceding to the formulation of polymeric nanoparticles, the physicochemical characteristics of drug i.e. NTZ was studied via several significant parameters viz. organoleptic evaluation (colour, odour, and texture), melting point range (Digital melting point apparatus), ultraviolet-visible (UV) spectroscopy and drug-excipient compatibility studies (FTIR). A standard solution (1µg/ml) of NTZ was prepared in methanol and scanned by UV-VIS spectrophotometer (Shimadzu UV-1800) between 200-800 nm. Furthermore, calibration curve was also plotted by the method suggested by Kapse et al [14]. NTZ was dissolved in 20 ml of methanol in a volumetric flask and treated with a solution (1gm of zinc granules in 10ml of 5N HCl) while shaking, kept at room temperature for 1h. After that, it was filtered through cotton wool, residue washed with 10ml portion of methanol three times and volume was made up to 100ml. Final concentration of reduced NTZ was made up to 100 µg/ml. From the stock solution, concentrations of 5 to 25µg/ml were prepared and measurement done by UV-visible spectrophotometer [14]. FTIR studies were directed by storing the drug, excipient and racemic mixture of drug and excipients (1:1) for 15 days at room temperature in separate glass vial. After that drug-KBr disc, excipient KBr disc and racemic mixture KBr disc (1:1) were exposed for scanning from 4000cm^{-1} to 400cm^{-1} using FT-IR spectrophotometer in a reflectance mode (Perkin Elmer Spectrum Rx, Serial No. -79225). The concept behind the selection criteria of polymer is that the Eudragit RL 100 is a positively charge polymer to synthesise nanoparticles which rises the interface between mucin and nanoparticles thus increases the drug bioavailability [8]. Due to biodegradable, aqueous solubility, minimal cell adhesion produces non -toxic effect, stability towards temperature variation and easy entanglement with nanoparticles surface PVA is selected as an another polymer [5,8].

2.2.2 Method of formulation development

In the present investigation the polymeric nanoparticles are prepared by homogenisation technique. An organic phase consist of polymer (Eudragit RL 100) and the model drug (NTZ) dissolved in acetone and was gradually added in organic phase. The above organic phase will added in to aqueous phase of PVA (Concentration varied according to optimisation) to form a mixture of nanoparticles using high speed homogeniser. The mixture was breakdown with the help of high speed blades at varying speed (2000-10000 rpm) for 2hrs. The stability of nanoparticles is achieved by continues stirring for removal of maximum solvent. The prepared formulation was centrifuged at 20,000 rpm for thirty minutes followed by collection of polymer based nanoparticles, dilution with double distilled water to be its immediate storage at 4□ under dark condition before characterisation [6,7].

We studied the effect of various processing parameters by BBD on particle size. The processing parameters include polymer concentration in organic phase, PVA

concentration in aqueous phase and speed of rotation of homogeniser. The different formulation are prepared by 3^3 factorial design and shown in Table 1.

2.2.3 Optimization of polymeric nanoparticles using factorial design (BBD)

The preferred independent variables were polymer concentration (%w/v) and cross-linking agent concentration (%w/v) and speed of rotation. For the NTZ loaded polymeric nanomaterials optimization, 3 factors, 3 levels were used. On the basis of information acquired from initial screening trials of different process variables and its three level (-α, 0, +α) in addition to low, medium and high, next narrow range optimisation was done by design expert ® Version 12 software using 3^3 factorial design. The preferred dependent variables were particle size (nm), PDI and zeta potential (mV). The design expert software is used to analyse 17 runs (12 main design points and 05 central points) with polymer concentration (1-5%), cross linking agent concentration (0.5-4.5%) and speed of rotation (2000-10,000 rpm) as independent variables. NTZ loaded nanoparticles were formulated and matching values of responses or variables were entered in the design. Ultimately to obtain the formulation of maximum desirability, constraints with their relevant importance were applied [3].

All formulation combinations in this study were prepared in triplicate.

The following equation (1) was produced for dependent variable.

$$Y=\alpha_0+\alpha_1X_1+\alpha_2X_2+\alpha_3X_3+\alpha_{12}X_1X_2+\alpha_{13}X_1X_3+\alpha_{23}X_2X_3+\alpha_{11}X_1^2+\alpha_{22}X_2^2+\alpha_{33}X_3^2 \quad \text{equation (1)}$$

Where Y is response variable; α_0 is intercept coefficient; α_1, α_2 and α_3 indicates linear coefficients; α_{11}, α_{22}, α_{33} are quadratic coefficients and α_{12}, α_{13}, α_{23} are interaction coefficients.

2.2.4 Evaluation parameters of polymeric nanoparticles

2.2.4.1 Particle size and PDI

The diameter of different preparations were measured with the help of Malvern zeta sizer. The size distribution was studied in terms of PDI. The procedure was performed by adding small amount of sample in to viewing unit of dynamic light scattering (DLS) at 25□ temperature with an angle with 173 degree is used to measure the particle size. And the diameter was determined by three parallel (n=3) measurement as an average unit and stated as mean ± standard deviation (SD). This technique measures the diffusion of particles under brownian motion and transform it into particle size and size distribution [14].

Table 1. Formulation table of polymeric nanoparticles using independent variables as per Box-Behnken design

Formulation code	Drug (mg)	Polymeric conc. (%)	Crosslinking agents (%)	Speed of Rotation (rpm)
F1	100	3	2.5	6000
F2	100	5	4.5	6000
F3	100	5	0.5	6000
F4	100	3	4.5	2000
F5	100	3	0.5	10000
F6	100	3	2.5	6000
F7	100	3	2.5	6000
F8	100	1	0.5	6000
F9	100	1	2.5	10000
F10	100	3	0.5	2000
F11	100	1	2.5	2000
F12	100	3	2.5	6000
F13	100	5	2.5	2000
F14	100	3	2.5	6000
F15	100	3	4.5	10000
F16	100	1	4.5	6000
F17	100	5	2.5	10000

2.2.4.2 Zeta potential measurement

The value of zeta potential was determined by Malvern zetasizer. In this technique electrophoretic kinesis was transformed to zeta potential of nanoparticles. The analysis was performed by diluting the sample with KCL (0.1mM) and then located in electrophoretic cell in the presence of 15.2V/cm electric field. All measurement of samples were carried out in triplet [14].

2.2.4.3 % Drug entrapment efficiency (DEE)

The nanoparticles entrapment efficiency of all formulations was determined by UV spectrophotometer. The accurately weighed quantity of lyophilized nanomaterial (2 mg) were mixed in 5 ml methanol, sonicated for 5 min. Then, the sample was added with 0.5ml of 2%v/v para-dimethyl amino benzaldehyde (PDAB) solution prepared in methanol [15]. Then, solution was heated for 10 minutes at 60-70°C temperature on water bath. After cooling, volume of sample solution was made up to 10 ml by methanol. Then, solution was divided in to 20 aliquots of 500 µl and individual aliquots was clarified through a milipore centricon filter membrane [16]. The nanoparticles were retained while free drug passed through the filter membrane. The amount of drug present in the filtrate was analysed by using UV-VIS spectrophotometer (Shimadzu, Japan) at 559 nm. The % drug entrapment efficiency (DEE) was calculated with the given formula:

% DEE = Actual drug content / Theoretical drug content × 100

Total readings were occupied in triplet (n= 3) and standard deviation (S.D.) was calculated from the average value (mean ± S.D.)

2.2.4.4 FTIR analysis studies

The FTIR analysis of freeze-dried polymeric formulation was attained using a FTIR spectroscopic instrument (Bruker, Model no.- Tensor 37, Jamia Milia Islamia, New Delhi). The KBr disc was scanned individually at 4 mm/s at a resolution of 2 cm over a range of 400–4,000 cm^{-1}. The distinctive peaks were recorded for different samples [17].

2.2.4.5 XRD analysis

X-ray diffractogram of pure drug sample, grafted polymer, PVA and polymeric nanoparticles (NPs) was recorded by HR resolution X-Ray Diffractometer (HRXRD), Jamia Milia Islamia, New Delhi by means of copper target and K-beta filter. The Powder sample was straddling on a plate which is made up of quartz and curved to a level of surface. At a scanning speed of 3° min−1 radiation the pattern of each sample through XRD was stately over an angular choice from 5° to 90° (diffraction angle 2θ) in stepwise raises of 0.05° [18].

2.2.4.6 Transmission electron microscopy (TEM)

The morphology of nanoparticles was studied by transmission electron microscopy (TEM) (JEOL JEM-1010S Tokyo, Japan). Firstly, the prepared formulation were diluted with distilled water and put a drop on to a holey carbon coated copper 400 mesh grid for forming a thin liquid film and the excess amount of suspension was absorbed by filter paper immediately. After that the liquid layer on grid were permitted to dry at room temperature and then detected in TEM instrument [19].

2.2.4.7 In-vitro drug release studies in simulated gastric fluid

The drug release studies of NTZ loaded polymeric nanoparticles (equivalent to 100 mg of drug) were performed by dialysis bag diffusion method. Drug laden nanoparticles (5 ml) were distributed in to dialysis bag and put this into a beaker containing 100 ml of phosphate buffer (7.4 pH). [20]. The beaker was placed over magnetic stirrer at 100 rpm under maintenance of 37±°C temperature throughout the experiment. Aliquots was withdrawn periodically and replaced with fresh medium to maintain sink condition throughout the period. Samples were withdrawn at predetermined time intervals using a pipette, the tip of which was covered with filter paper to avoid drug particles [21]. The withdrawal samples were treated with 0.5ml 2% w/v methanolic solution of para-dimethyl amino benzaldehyde (PDAB) solution. All samples were kept for 10 minutes at 60-70°C temperature on water bath. NTZ content in the aliquots after that adding of PDAB solution was assayed through UV-VIS spectrophotometer at 559 nm for all samples. The experiments were done in triplicate (n=3) and standard deviation (SD) was computed from the mean value.

2.2.4.8 Well diffusion agar method

Nutrient agar media

1 litre of nutrient broth (HiMedia-GRM666-500G) was prepared by dissolving 13g of commercially available nutrient broth in 1000ml distilled water, then agar agar 1 (Lot-GRM666) (2 gm/1000ml) was added and heated to dissolve the medium entirely. The media was decontaminated by autoclaving at 15lbs pressure (121°) for 15 minutes (Autoclave Model no. IS 4159).

Method of antimicrobial assay

S. mutans (MTCC-3160), *E. coli* (MTCC-1563), *P. aeruginosa* (MTCC-2453) were used to calculate antimicrobial action of optimised formulation by well diffusion agar method. In a sterile condition wells were cut and 20 µl of the given samples (of different concentrations) were added. The plates were then incubated at 37° for 24 hours. Zone of inhibition diameters arisen around the discs were measured, verified and the results were calculated [22].

3. RESULTS AND DISCUSSION

3.1 Preliminary Formulation Study

NTZ was found to be yellow crystalline powder which showed melting point with in the range of 199±1°C to 201±1°C as reported, thus signifying purity of sample. The UV spectrum of NTZ shown the absorption maxima at 559 nm in methanol with further derivatization using p-dimethyl amino benzaldehyde (PDAB). Hence, all further UV estimation was done at maximum wavelength 559 nm. Calibration curve was plotted with series of dilutions. The regressed equation is y = 0.0059x – 0.0054. The value of r^2 is close to 1 i.e. 0.9953. The FTIR spectra of pure drug (NTZ), Eudragit RL 100 and racemic mixture of NTZ , Eudragit RL 100 and PVA (1:1:1) confirmed the absence of any chemical interaction between them after 15 days of samples storage**.** The values of functional group reported through FTIR spectrum as shown in Fig. 5.

3.2 Screening of Factors and Variables

Firstly, lowest amount of Eudragit RL 100 polymer was determined which would be sufficient concentration for the preparation of polymeric nanoparticles at room temperature. So, we found that below 1% polymer concentration the formation of polymeric nanoparticles does not proceed while above 5% of polymeric concentration nucleation mediated size development of nanoparticles in the formulations which could be produced immobility of polymeric nanoparticles in the solution due to growth in the viscosity of polymeric solution . We further screened out three critical factors including polymeric concentration, cross linking agent concentration, cross linking agent concentration and homogeniser speed with the help of design expert software version 12.0 which showing maximum effect on anticipated attributes of formulation. While other statistically irrelevant procedure variables like temperature was fixed throughout the experiments. In case of PVA concentrations we found that below 0.5 % the crosslinking phenomenon with polymer was low, while at above 4.5% the size of nanoparticles enhances due to adherence of PVA on the surface of prepared formulation of polymeric nanoparticles. The increase in particle size has been also reported as concentration of PVA enhanced [23,24]. The speed of rotation of homogeniser depends upon the phenomenon of dynamism density that is energy applied per unit volume through the blade is directly produces effect on particle size of nanoparticles. The scientific reason behind it is that the magnitude of shear stress is inversely proportional to the particle size of polymeric nanoparticles. Therefore the formulation of nanoparticles occurs at a range of speed of rotation of homogeniser (2000-10,000 rpm) for approx 45 minutes except evaporation time [25]. The levels of each of these variables, need of BBD under which the combinations of consideration of all factors would produces the polymeric nanoparticles during experimental procedure are represented in Table 2.

The design expert software is used to predict the 17 positive runs which involved the centre of each edge and replicated the centre points for optimisation of polymeric nanoformulation. The prepared formulations were characterised for the dependent variable like nanoparticle size, PDI and zeta potential and the results were shown in Table 2. Polynomial equations which explained the interaction effect, individual main and quadratic effect of the considered independent runs variables revealed the significant effect on observed responses. The result of evaluation for an individual response were studied by ANOVA and were shown in Table 3. The figure of the 3-dimensional response surface plots (RSP) were collaborated to interpret the effects of

independent variables on the values of dependent responses. This investigation assist in calculating the ideal set of investigational parameters [26].

Table 2. Impact of three factors, at their three different levels, on final performance as per the Box—Behnken design

Formulation code Runs (F1-F17)	Independent variables			Dependent variables		
	Polymeric conc. (%)	Cross linking agents (%)	Speed of Rotation (rpm)	Particle Size (nm)	PDI	Zeta Potential (mV)
F1	0	0	0	152.05±2.31	0.310±0.01	25.9±4.87
F2	+1	+1	0	165.18±4.04	0.490±0.01	6.11±5.77
F3	+1	-1	0	148.38±1.78	0.254±0.08	28.5±3.39
F4	0	+1	-1	164.98±1.66	0.430±0.12	9.29±4.52
F5	0	-1	+1	146.05±3.11	0.242±0.07	29.6±5.01
F6	0	0	0	152.05±2.31	0.310±0.01	25.9±4.87
F7	0	0	0	152.05±2.31	0.310±0.01	25.9±4.87
F8	-1	+1	0	141.80±2.02	0.183±0.06	32.4±3.15
F9	-1	0	+1	143.14±1.14	0.223±0.07	30.4±4.89
F10	0	-1	-1	151.71±2.57	0.286±0.03	26.8±4.33
F11	-1	0	-1	148.81±2.45	0.267±0.02	27.6±3.44
F12	0	0	0	152.05±2.31	0.310±0.01	25.9±4.87
F13	+1	0	-!	155.38±1.21	0.352±0.08	17.3±2.76
F14	0	0	0	152.05±2.31	0.310±0.01	25.9±4.87
F15	0	+1	+1	159.31±3.11	0.381±0.18	15.0±5.23
F16	-1	+1	0	155.07±2.65	0.344±0.09	18.4±4.38
F17	+1	0	+1	153.25±2.13	0.325±0.02	20.2±5.77

Table 3. ANOVA for the response surface quadratic model for particle size, PDI and zeta potential

Response 1: Particle Size (Y_1)								
Source	Sum of Squares	df	Mean Square	F-value	p-value	Model summary statistics		
						R^2	Adj. R^2	Pred. R^2
Model	650.97	9	72.33	162.20	< 0.0001	0.9952	0.9891	0.9236
Polymeric concentration (X_1)	139.22	1	139.22	312.21	< 0.0001			
Cross linking agent (X_2)	400.25	1	400.25	897.59	< 0.0001			
Speed of rotation (X_3)	45.77	1	45.77	102.64	< 0.0001			
(X_1,X_2)	3.12	1	3.12	7.00	0.0331			
(X_1,X_3)	3.12	1	3.12	7.00	0.0332			
(X_2,X_3)	0.0000	1	0.0000	0.0000	1.0000			
$(X_1)^2$	24.35	1	24.35	54.61	0.0002			
$(X_2)^2$	36.94	1	36.94	82.84	< 0.0001			
$(X_3)^2$	1.05	1	1.05	2.36	0.1682			
Residual	3.12	7	0.4459					
Lack of Fit	3.12	3	1.04					
Pure Error	0.0000	4	0.0000					
Cor Total	654.09	16						

Response:2 Polydispersivity index (PDI) (Y_2)								
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	**Model summary statistics**		
						R^2	**Adj. R^2**	**Pred. R^2**
Model	0.0869	9	0.0097	37.59	< 0.0001	0.9797	0.9537	0.6757
Polymeric concentration (X_1)	0.0204	1	0.0204	79.45	< 0.0001			
Cross linking (X_2)	0.0578	1	0.0578	225.09	< 0.0001			
Speed of rotation (X_3)	0.0034	1	0.0034	13.09	0.0085			
(X_1,X_2)	0.0014	1	0.0014	5.48	0.0518			
(X_1,X_3)	0.0001	1	0.0001	0.2814	0.6122			
(X_2,X_3)	6.250E-06	1	6.250E-06	0.0243	0.8804			
$(X_1)^2$	0.0013	1	0.0013	5.09	0.0586			
$(X_2)^2$	0.0027	1	0.0027	10.56	0.0141			
$(X_3)^2$	1.645E-06	1	1.645E-06	0.0064	0.9385			
Residual	0.0018	7	0.0003					
Lack of Fit	0.0018	3	0.0006					
Pure Error	0.0000	4	0.0000					
Cor Total	0.0887	16						
Response: 3 Zeta Potential (Y_3)								
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	**Model summary statistics**		
						R^2	**Adj. R^2**	**Pred. R^2**
Model	887.71	9	98.63	123.32	< 0.0001	0.9937	0.9857	0.8997
Polymeric concentration (X_1)	168.27	1	168.27	210.38	< 0.0001			
Cross linking agent (X_2)	586.53	1	586.53	733.32	< 0.0001			
Speed of rotation (X_3)	25.24	1	25.24	31.56	0.0008			
(X_1,X_2)	17.60	1	17.60	22.00	0.0022			
(X_1,X_3)	0.0025	1	0.0025	0.0031	0.9570			
(X_2,X_3)	2.12	1	2.12	2.65	0.1478			
$(X_1)^2$	0.7516	1	0.7516	0.9397	0.3646			
$(X_2)^2$	71.64	1	71.64	89.57	< 0.0001			
$(X_3)^2$	10.81	1	10.81	13.52	0.0079			
Residual	5.60	7	0.7998					
Lack of Fit	5.60	3	1.87					
Pure Error	0.0000	4	0.0000					
Cor Total	893.31	16						

3.3 Formulation Optimisation Using BBD

BBD is used to design 17 formulations by 3^3 factorial design for optimised formulation of polymeric nanoparticles. In this investigation, two formulation variables and one process variables was selected with three response at three levels of dependent variables. Result of ANOVA, in which different parameters are discussed like type of model, coefficient of correlation, probability of particle size (Y_1), PDI (Y_2), and zeta potential (Y_3) respectively. Furthermore, outcomes of proposed design that the system was significantly influenced by the concentration of polymer (X_1), cross linking agent (X_2) and speed of rotation (X_3); affecting particle size , PDI and zeta potential for the formulation of nanoparticles. ANOVA was utilized to estimate model significance and their quantitative effects on respective responses. Response surface curves were plotted for optimisation of nanoparticles as shown in Fig. 1-3. The 3-D model curves of different parameters were employed to elucidate the interaction of the factors with responses [27].

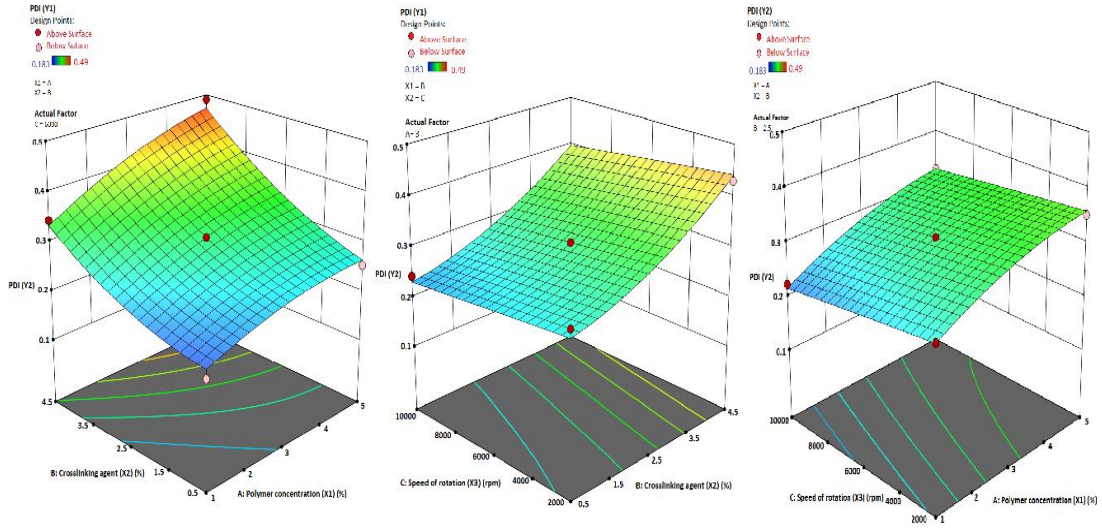

Fig. 1. 3D Response surface graph representing role of various factor on particle size

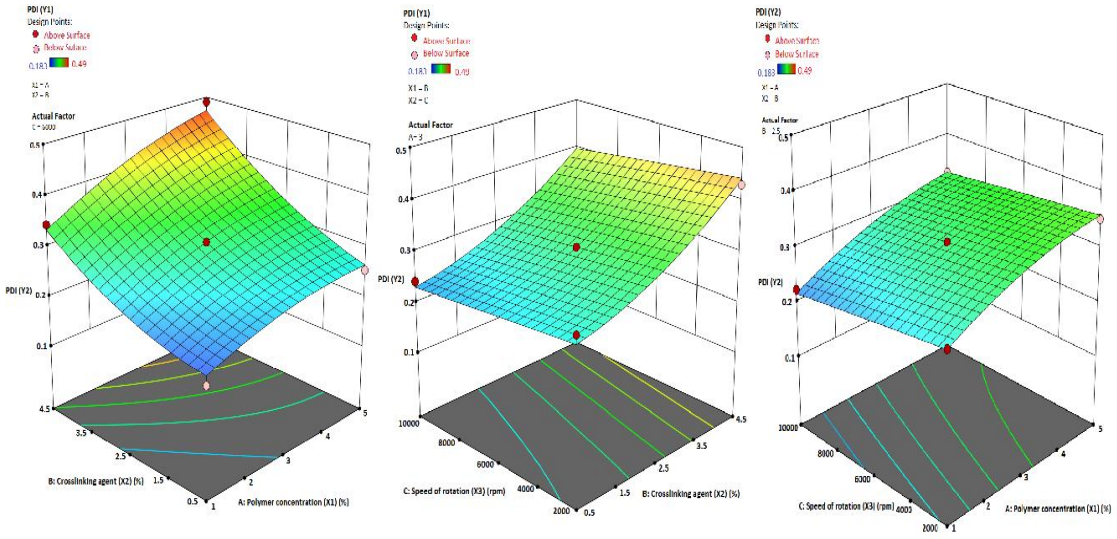

Fig. 2. 3D Response surface graph representing role of various factor on PDI.

3.4 Particle Size

Decrease in particle size, especially at nanometre size exhibit considerable consequence on release of drug**.** Hence, reduction in particle size of polymeric nanoparticles may have a helpful effect in absorption due to increase in surface area. Average particle size varied from 141.80 nm to 165.18 nm, which is affected by concentration of polymer, cross linking agent and speed of rotation (Table 2). Statistical analysis of data showed that the model proposed (quadratic) was significant (p<0.05), with F-value (162.20) and a high coefficient of correlation (R^2=0.9952) as shown in Table 3. Resultant polynomial equation (2) for the fitted model related to the particle size is as follows:

$$Y_1 = 152.05+4.17X_1+7.07X_2-2.39X_3+0.88X_1X_2+0.88X_1X_3+0.00X_2X_3-2.40X_1^2+2.96X_2^2+0.50X_3^2 \quad \text{equation (2)}$$

Adjusted and predicted R^2 of quadratic model forecasting particle size were 0.9891 and 0.9236, respectively as shown in Table 3. Single effects (X_1), (X_2) (X_3), interaction terms (X_1X_2), (X_1X_3) and quadratic terms X_1^2 and X_2^2 showed a

significant effect (p<0.05), with large coefficients (equation 3). Nonetheless, X_3^2 and (X_2X_3) has a non-significant influence (p>0.05) on particle size (Y_1). On increasing the concentration of polymer, the particle size increases. This produce (positive) effect on response Y_1 was because of a higher concentration of polymer in organic phase, which increases viscosity, and results into a larger particle size as shown in Fig. 1 [28,29]. On increasing crosslinking agent concentration (X_2), an insignificant increase in particles size was observed. Speed of rotation (X_3) has negative effect on particle size. An increased speed of rotation bring system in enhanced shear rate, which eventually breaks the larger globules of polymer solution into smaller one, resulting into a considerable reduction in particle size. Positive coefficient of binary interaction term (X_1X_2), in mutual setting, suggested that the mixed effect of concentration of Eudragit RL 100 and PVA has a significant increase in average particles size. This might be due to the formation of mixed micelle [28,30]. A positive coefficient of the binary interaction (X_1X_3) and (X_2X_3) construed that the mixed effect of the polymeric concentration and speed of rotation, PVA concentration and speed of rotation, showed an increase in average particles size [31]. Here, the effect of polymer and PVA was dominated over stirring speed. Quadratic effect (X_1^2), of polymer gave a small negative coefficient (with a *p>0.05*), interpreting an insignificant effect on particle size. Excess addition of polymer may offer micelle formation, which eventually results into reduction of average particle size. On the other side, quadratic effect of amount of polymer (X^2_2) has a higher positive and significant influence (p<0.05) as compared to single effect (X_2), which exhibited a considerable increase in particle size. This effect might be due to overcrowding (multiple layering) of surfactant molecules. Quadratic effect of stirring speed (X^2_3) has a non-significant effect (*p<0.05*), although it produces negative effect as single effect (X_3) showed a decrease in average particle size. At higher rpm (rotation speed), a forceful, uniform, and increased mechanical shear may have resulted, which means that efficiency of mixer is no longer enhanced under given set of conditions with an increase in rpm [32]. The Model F-value 162.20 indicates the model is significant. There is only a 0.01% chance that an F-value found to be large might happen due to sound.

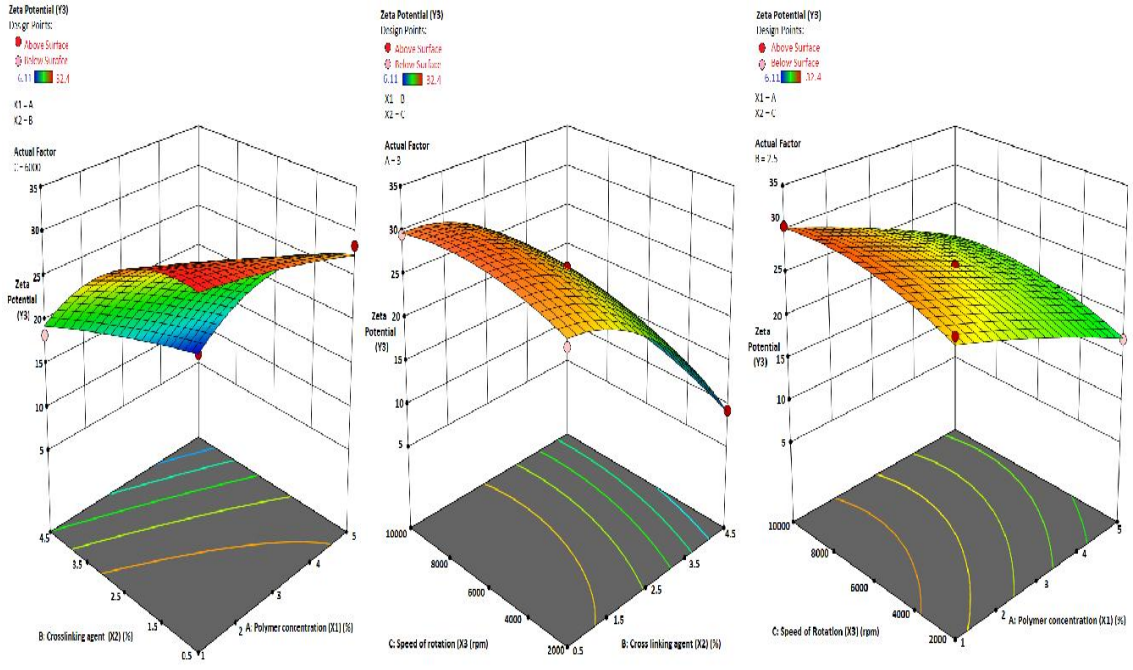

Fig. 3. 3D Response surface graph representing role of various factor on zeta potential

3.5 Particle Size Distribution

In this setup, PDI is used as a key factor for quantitation of width of particle size distribution of polymeric nanoparticles. PDI ranges from 0.183 to 0.490, and it was influenced by concentration of polymer (X_1), concentration of crosslinking agent (X_2) and speed of rotation (X_3) (Table 2). Statistical analysis of data showed that proposed model (quadratic) was significant (p<0.05) and results into a high F-value (37.59) with coefficient of correlation (0.9797). An evaluation between actual PDI (observed value) and software generated predicted values, exhibited a concord. The 3-D response surface curves of PDI (Y_2) in relation to X_1 and X_2; X_1 and X_3; X_2 and X_3 are exhibited in Fig. 2 obtained for fitted model related to PDI and independent variables are as follows:

$$Y_2=0.31+0.050X_1+0.085X_2-0.020X_3+0.018X_1X_2+0.004X_1X_3-0.001X_2X_3-0.017X_1^2+0.0254X_2^2-0.0006X_3^2 \quad \text{equation (3)}$$

Adjusted and predicted R^2 of quadratic model forecasting PDI were 0.9537 and 0.6757, respectively as shown in Table 3. Single effects (X_1), (X_2), (X_3) and quadratic terms (X_2^2) showed a significant influence (p<0.05). Whereas interaction terms (X_1X_3), (X_2X_3) (X_1X_2) and quadratic terms (X_1^2), (X_3^2) showed a non-significant influence (p>0.05). Single effect of Polymer (X_1) on formulation is positive with respect to PDI (equation 3). The lower concentration of polymer in organic phase broken down into uniform globules as much as possible, which finally results into lower PDI therefore on increasing an amount of polymer resulted in an increase in PDI as shown in Fig. 2. The same profile were observed in case of first dependent variable. PDI increases on increasing the concentration of PVA from 0.5% to 4.5% due to deposition of PVA on the surface of polymeric nanoparticles. Single effect (X_1), (X_2) exhibited a significant positive effect on PDI. [32]. On the other hand, speed of rotation (X_3) exhibited a significant (p<0.05) and negative effect on PDI. Shearing caused by stirring bring polymeric organic phase solution into globules of varied size, resulting in a significant increase in PDI. Low stirring rate might have diminished uniformity of mixing force throughout volume of nanoformulation therefore resulted into an increased PDI [33,34]. Quadratic effect (X_1^2) of polymer resulted into a negative coefficient (with a p>0.05), construing a broadening in PDI. It was occurs because of enhanced viscosity of polymer solution [28]. Quadratic effect of PVA concentration (X^2_2) has a higher positive and significant influence (p<0.05) as compared to single effect (X_2), which showed a considerable decrease in PDI. The quadratic effect of stirring speed (X^2_3) has a negative and significant (p>0.05) effect, which exhibited an increase in PDI. The Model F-value 37.59 indicates the model is significant. There is only a 0.01% chance that an F-value found to be large might happen due to sound.

3.6 Zeta Potential

In this setup, zeta potential is used as a key factor for determining stability of nanoparticle size distribution of polymeric nanoparticles. Zeta potential ranges from 6.11 to 32.4mv, and it was influenced by concentration of polymer (X_1), concentration of crosslinking agent (X_2) and speed of rotation (X_3) (Table 2). Statistical analysis of data showed that proposed model (quadratic) was significant (p<0.05) and results into a high F-value (123.32) with coefficient of correlation (0.9937). An evaluation between actual zeta potential (observed value) and software generated predicted values, exhibited a concord. The 3-D response surface curves of zeta potential (Y_3) in relation to X_1 and X_2; X_1 and X_3; X_2 and X_3 are exhibited in Fig. 3. Equation (4) obtained for fitted model related to zeta potential and independent variables are as follows:

$$Y_3=25.90-4.59X_1-8.56X_2+1.78X_3+-2.10X_1X_2+0.025X_1X_3+0.727X_2X_3-0.422X_1^2-4.13X_2^2-1.60X_3^2 \quad \text{equation (4)}$$

Adjusted and predicted R^2 of quadratic model forecasting zeta potential were 0.9857 and 0.8997, respectively as shown in Table 3. Single effects (X_1), (X_2), (X_3), interaction terms (X_1X_2) and quadratic terms (X_2^2) showed a significant influence ($p>0.05$). Single effect of Polymer (X_1) and cross linking agent (X_2) on formulation is negative with respect to speed of rotation (X_3) (equation 4).On increasing the concentration of polymer the and cross linking agent the value of zeta potential is also increases [35] as shown in Fig. 3.

In an exact liquid media, zeta potential is used to calculate the charge on the surface of a particle. The determination of surface charge by zeta potential method supports in understanding and forecasting connections between particles of

suspension [18]. The degree of zeta potential is an indicator of the potential stability of colloidal system. The value of zeta potential is ±30 mV could appropriately contribute to the physical stability of suspension (27). The exterior of nanoparticles spreads a +ve charge due to of presence of the quaternary (4^{o}) ammonium (NH_3^+) groups on polymer [36].The Model F-value of zeta potential is 123.32 suggests the model is significant. There is only 0.01% chance that an F-value become high due to noise production.

3.7 Desirability and Validation of the Model

The desirability measures and response surface as well as contour plots of BBD, achieved from Design-Expert software 12, were facilitated to find the optimised formulation of polymeric nanoparticles the combination of independent variables from 17 runs with their contour representation of optimisation chart with highest desirability score among all suggested solutions as Fig. 4. The desirability score after optimisation was found to be nearest to 1 i.e. 0.999, which implied accurate result of the investigation. The optimized values of final formulation (NTZ Polymeric nanoparticles) lay between the selected ranges mentioned in Table 4. The aim of the optimization was to obtain minimum particle size, minimum PDI and maximum zeta potential. The numerical optimization was achieved using Design Expert software V.12.0 Hence, to authenticate the design model which were set to found polymeric nanoparticles, a comparative analysis between the final product performances with the theoretical was carried out through point prediction investigation of the given parameters. The optimization technique created predicted effects established on the prearranged values of the dependent variables as shown in Table 4 [23, 30]

The optimised polymeric formulation was found to be 1% of polymer, 0.5% concentration of crosslinking agent (PVA), and 9000 speed of rotation.

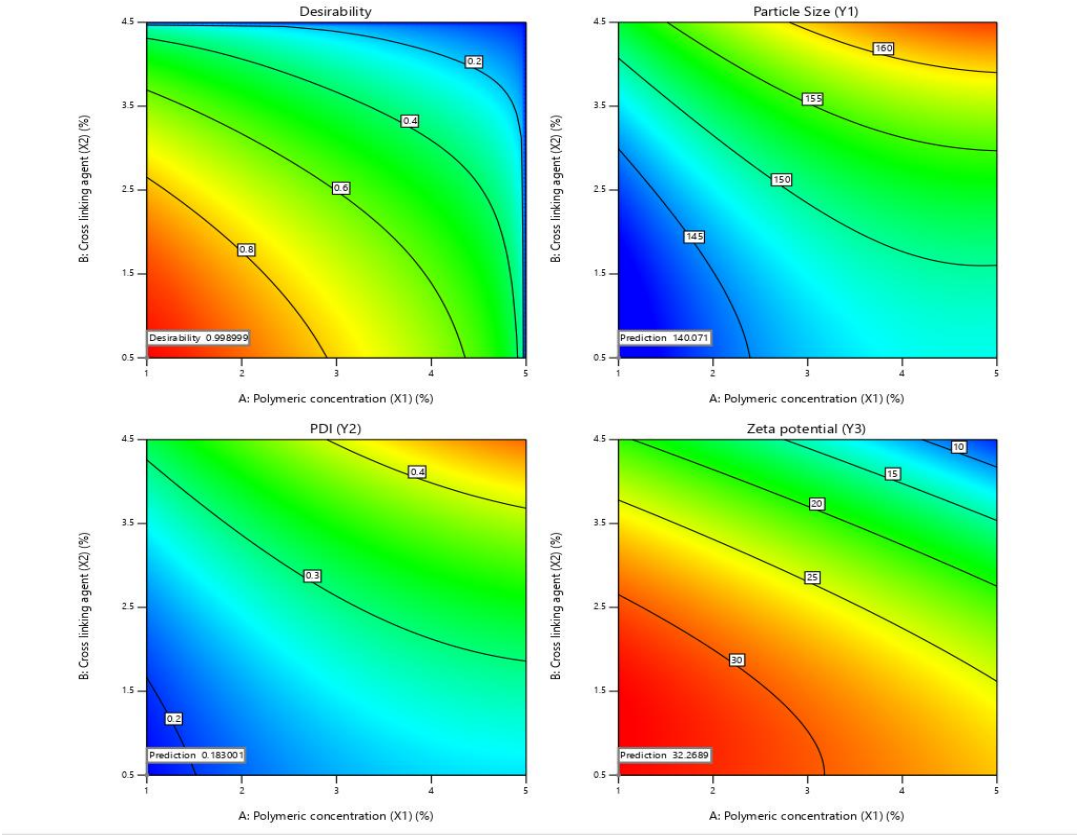

Fig. 4. Representation of desirability and validation of the model by contour plots

Table 4. Variables and their levels studied in Box-Behnken design

Independent Variables		Variables		
		-1	0	+1
(X_1)	Polymer concentration	1	3	5
(X_2)	PVA concentration	0.5	2.5	4.5
(X_3)	Speed of Rotation	2000	6000	10000
Dependent Variables		**Constraints**		
(Y_1)	Particle size	Minimize		
(Y_2)	PDI	Minimize		
(Y_3)	Zeta Potential	Maximize		

3.8 Evaluation of Optimised Formulation

3.8.1 Particle size, PDI and Zeta potential

It was observed that particle size of optimised formulation was found to be 137.11 nm and PDI was 0.180 after using optimisation parameter in BBD software. The value of zeta potential was predicted as 33.4mV as shown in Fig. 8. the values of response variables were selected on the basis of different goals of dependent and independent variables as shown in Table 5 The value of zeta potential shows the stability of polymeric nanoparticles, the presence of positive surface charge density on nanoparticles prevent the agglomeration process due to repulsion between particles with similar electric charge [37].

3.8.2 Drug Entrapment Efficiency

From the results, it was found that polymeric nanoparticles possess drug entrapment efficiency (%DEE) in the range of 44.4%±0.15 to 79.40%±0.48 . As the polymer concentration increased the entrapment efficiency also increased. On increasing the polymeric concentration, the viscosity of organic phase is also enhanced, which result in rise in viscous force resisting polymer breakdown and thus larger nanoparticles are made, resulting in increase in particle size and PDI. Due to rise in viscosity of organic solvent there is increase in diffusional opposition to drug molecules from organic part to aqueous part there by entangling high drug molecules in the polymeric nanoparticles [18]. Higher concentration of PVA also increases the encapsulation of drug in the nanoparticles but which further contributed to burst release in place of sustained drug release from the formulation [38]. The encapsulation efficiency increased from 44.4%±0.15 to 79.40%±0.48 when the speed of rotation of homogenizer was changed from 2000 rpm to 10,000 rpm. The high drug encapsulation occurs due to unidirectional and a lesser amount of turbulent flow in the case of lesser speed may have resulted in the loss of drug from the organic phase. The % DEE of optimised formulation was found to be 81.89%.

Table 5. Comparison of the predicted and experimental values of the response variables of optimized formulation

Dependent variable	Goal	Lower	Upper	Importance	Predicted value	Observed value	% Prediction error
Polymer concentration (x_1)	Minimum	1%	5%	3	1%		
PVA concentration (x_2)	Minimum	0.5%	4.5%	3	0.5%		
Speed of Rotation (x_3)	Within range	-1	+1	3	9000.3		
Particle size (Y_1)	Minimum	141.807	165.18	3	141.80	137.11	-0.028
PDI(Y_2)	Minimum	0.183	0.49	3	0.183	0.180	-0.016
Zeta Potential (Y_3)	Maximum	6.11	32.4	3	32.4	33.4	0.034

3.8.3 FTIR characterisation

The compatibility studies between drug and excipients was examined through the racemic mixture (1:1) and drug-loaded polymeric nanoparticles by FTIR spectroscopy. The spectra of pure drug sample (NTZ), Eudragit RL 100, PVA, NTZ-Eudragit-RL100-PVA racemic mixture, Placebo (NPs) NTZ-loaded polymeric formulation were observed in a range of 3600 to 400 cm−1 and presented in Fig. 5. The distinctive peaks of NTZ were identified at 3350.20 cm−1 due to aromatic N–H stretching, 1520.34 cm−1 due to C–N-H bond stretching, 1771.04 cm−1 due to the carbonyl group (C=O) and 1465.35 cm−1 due to C=C aromatic group, 1355.35 cm−1 due to N=O stretching attach to the aromatic ring. The spectra of Eudragit RL 100 showed distinctive peaks at 1053.80 cm−1 due to C-O stretching, 2969.93 cm−1 due to C–H stretching, and 1015.02 cm−1 (C–N stretching vibration). In case of PVA the presented lesser intense peaks (3374.83 cm−1 for O–H stretching in molecular hydrogen bonds, 2778.73 cm−1 for stretching of C–H from alkyl groups), 1058.78 cm−1 due to C-O stretching of PVA which definite effective elimination of surfactant from nanoparticles [39]. The earlier labelled distinctive peaks of NTZ were also detected in racemic mixture and nanoparticles. There were no occurrence of main shifts in the distinctive peaks of NTZ and excipients which showed that there was no indication of major chemical interface among them. The result established the chemical reliability of the distributed drug molecules into polymeric nanoparticles containing excipients.

3.8.4 XRD analysis

The X-ray diffraction spectra showed the amorphous nature of polymer Eudragit RL 100 and PVA as shown in Fig. 6 as no characteristics peak was observed. The XRD spectra of pure drug (NTZ) confirmed its crystalline nature as shown spectra (Fig. 6). However, the XRD spectra of grafted excipients shows less intense peak but crystalline, which confirmed the grafting of drug on to the surface of polymer which have also confirmed by literature [39,40].

3.8.5 TEM of optimised formulation

The optimised polymeric nanoparticles were further characterised by TEM and concluded that prepared nanoparticles were spherical and uniform with size range in the acceptable limit as shown in Fig. 7. TEM images would provide an improved appreciative of the geometric particles size and the relationship among the process variables and particle size [7].

3.8.6 *In-vitro* release profile

All formulations revealed an initial burst release as shown in Fig. 9 .This may be attributed to the drug leaching from the outer layer of polymeric nanoparticles with subsequent entry of dissolution fluid inside the polymer matrix resulting dissolution and diffusion of drug upto 2 hrs, 22.34%±0.14 to 35.12±0.28 release has been observed for all formulations. In the 1st hour the release profile was found to be in the range of 18.44%±0.14 to 31.44±0.18% for all

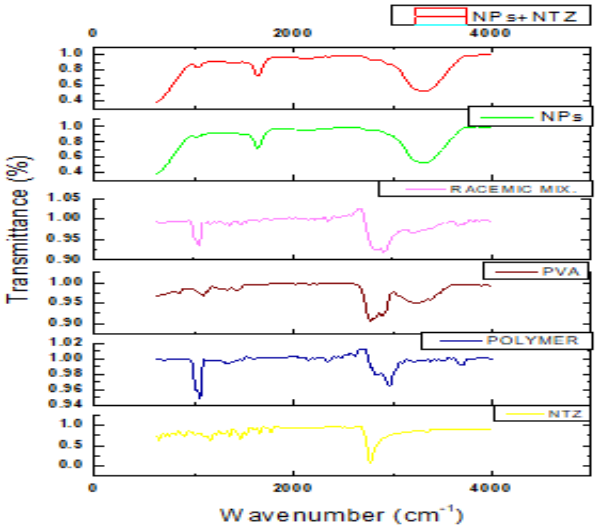

Fig. 5. FTIR graph for estimation of compatibility studies

formulations respectively. Preliminary burst drug release happened for the reason that the drug molecules might be present on the exterior layer of nanoparticles. After the 1st hour the release of drug molecule from the nanoparticles were released gradually. Due to the presence of large number of acidic moieties on the polymeric backbone the drug release need a higher pH to attain the maximum degree of ionisation for the dissolution of polymer. Thus release profile of formulation was carried out in phosphate buffer (pH 7.4) [41]. The maximum *in-vitro* release was found to be 86.88% for formulation F8 after 24 hrs because F8 contains minor polymer concentration and PVA. And the minimum *in-vitro* release profile was found to be 60.23% through F3 formulation after 24 hrs among all F17 runs because F3 contains high amount of polymer and PVA. [39,40,42]. And the *In-vitro* drug release of optimised formulation was found to be 88.89% which showed sustained drug release profile.

3.8.7 Antimicrobial activity estimation

The average zone of inhibition for various samples against different bacteria's were shown in Table 6.

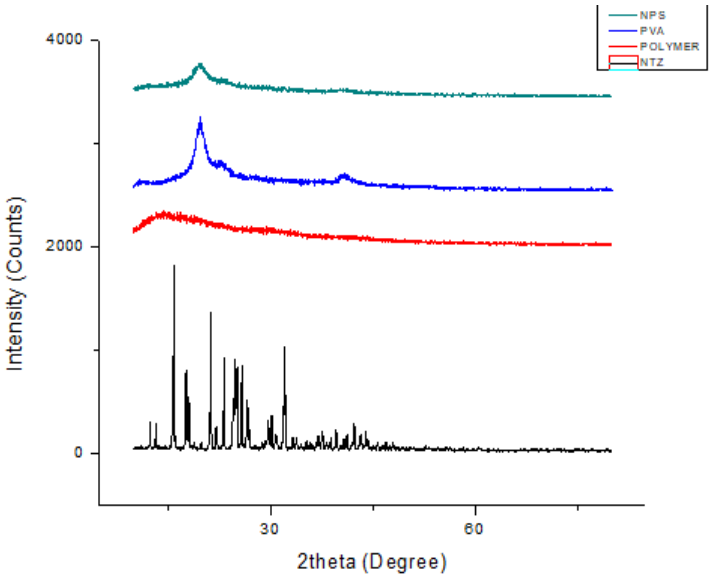

Fig. 6. XRD representation of pure drug sample, excipients and NPs

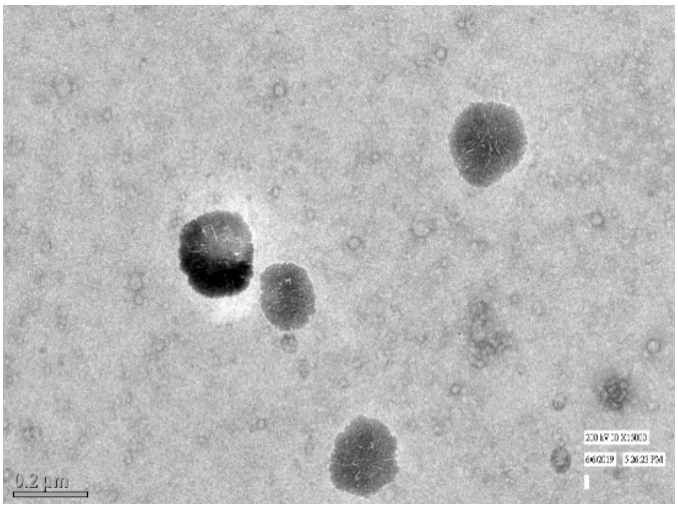

Fig. 7. TEM image of optimised formulation

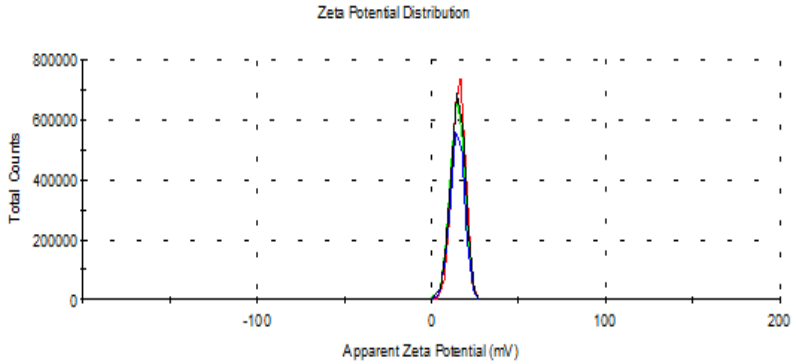

Fig. 8. Zeta potential graph of optimised formulation

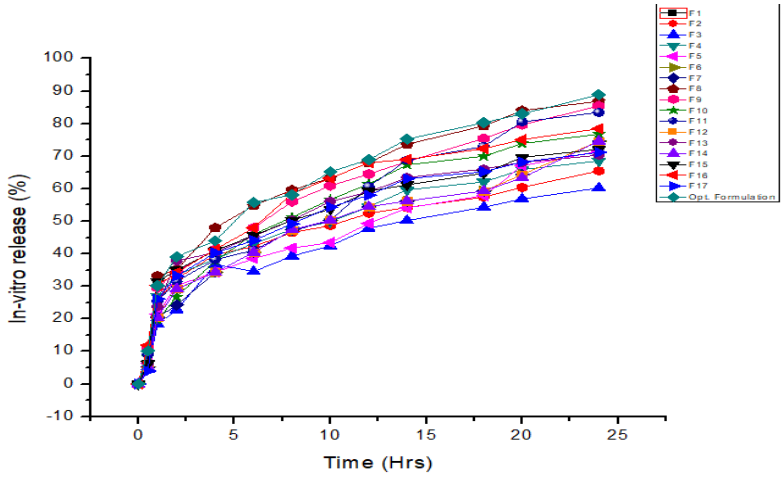

Fig. 9. *In-vitro* release profile of all formulations according to BBD including optimised formulation

Table 6. Average zone of inhibition of various samples against different bacteria's

S.No.	Various samples in different concentration (µg/ml)	Average Zone of inhibition (mm)		
		Streptococcus mutans	***Escherichia coli***	***Pseudomonas aeruginosa***
1.	Metronidazole (Std)			
	16 µg/ml	10.33±0.33	03±0.34	08.13±0.16
2.	Nitazoxanide NTZ			
	14µg/ml	07.17±0.236	00±00	06.33±0.55
	16µg/ml	13.03±0.05	04±0.96	09.13±0.20
3.	NTZ+NPs			
	10 µg/ml	00±00	00±00	06.58±0.66
	15 µg/ml	07.10±0.860	05.96±0.25	11.76±1.56
	20 µg/ml	17.93±1.636	06.30±0.294	13.48±0.843

In first case the standard concentration was 16 µg/ml, however the average zone of inhibition was 10.33, 03.00 and 08.13 against microbes *S. mutans*, *E. coli* and *P. aeruginosa* (Table 6 and Fig. 10). In second sample (NTZ) concentrations were taken of 14 µg/ml and 16 µg/ml. The average zone of inhibition was 7.17 and 13.10 with both concentrations against *S. mutans.* The average zone of inhibition was 6.33 and 9.13 with both concentrations against *P. aeruginosa*. While no zone of inhibition was appeared against *E. coli* at 14 µg/ml while at 16 µg/ml the zone of

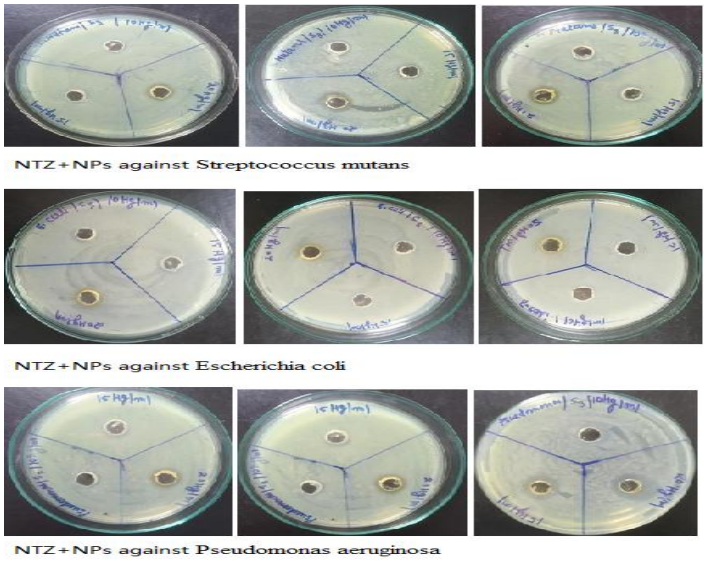

Fig. 10. Antimicrobial activity of optimised formulation (NTZ+NPs)

inhibition was found to be 04.00. *E. coli* showed the least zone of inhibition due to maximum resistant capability of the bacterial isolates. The zone of inhibition of model drug against different bacteria's is higher than the standard drug [43]. In sample III (NTZ+NPs) evaluation was done with three concentration 10µ g/ml 15 µg/ml and 20µg/ml. The average zone of inhibition was 00.00, 07.10 and 17.93 with increase concentration of sample (10 µg/ml 15 µg/ml and 20 µg/ml) against microbes *S. mutans.* The average zone of inhibition was 00.00, 05.96 and 06.30 with increase concentration of sample (10 µg/ml 15 µg/ml and 20 µg/ml) against microbes *E.coli.* The average zone of inhibition was 06.58, 11.76 and 13.48 with increase concentration of sample (10 µg/ml 15 µg/ml and 20 µg/ml) against *P. aeruginosa* against control. After the drug incorporation was occurred in polymeric nanoparticles, it produced additive effect against antimicrobial activity.

4. CONCLUSION

In the above research work we synthesised the polymeric nanoparticles by homogenisation technique. After the identification of independent variables through conventional method we estimate the polymeric concentration (%), crosslinking agent concentration (%) and speed of rotation of homogeniser (rpm) as a probable independent factors for the selection of optimised formulation through their effects on dependent variables with the aid of BBD. Multivariate investigational design projected a quadratic model as the efficient relationship between the dependent variables (i.e particle size, PDI, zeta potential) and stated by response surface methodology. The characteristics of the formulated nanoparticles are attractive for pharmaceutical use as they showed high %DEE, narrow particle size distribution, good compatibility studies and prolonged drug release profile. The optimised polymeric formulation of spherical in size which was further confirmed by TEM analysis, while X-ray diffraction (XRD) to justify the amorphous and crystalline nature of drug and excipients. With the help of point prediction analysis the optimised formulation was synthesised and its particle size, PDI and zeta potential were found to be 137.11nm, 0.180 and 33.4 mV respectively. From the results, it is concluded that independent variables produced a significant effect on the measured dependent variables responses ($p < 0.05$). So, on the behalf of this complete investigation, novel NTZ loaded polymeric nanoparticles could be a potential drug delivery system for sustained effect and targeted release in the treatment of microbial infection.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for

any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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