Accepted Manuscript

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PII: S0141-8130(17)30480-4
DOI: http://dx.doi.org/doi:10.1016/j.ijbiomac.2017.05.129
Reference: BIOMAC 7618

To appear in: *International Journal of Biological Macromolecules*

Received date: 6-2-2017
Revised date: 10-5-2017
Accepted date: 21-5-2017

Chemical compounds studied in this article

Tetrachloroauric Acid (28133)

Gum ghatti mediated, one pot green synthesis of optimized Gold nanoparticles: Investigation of process-variables impact using Box-Behnken based statistical design.

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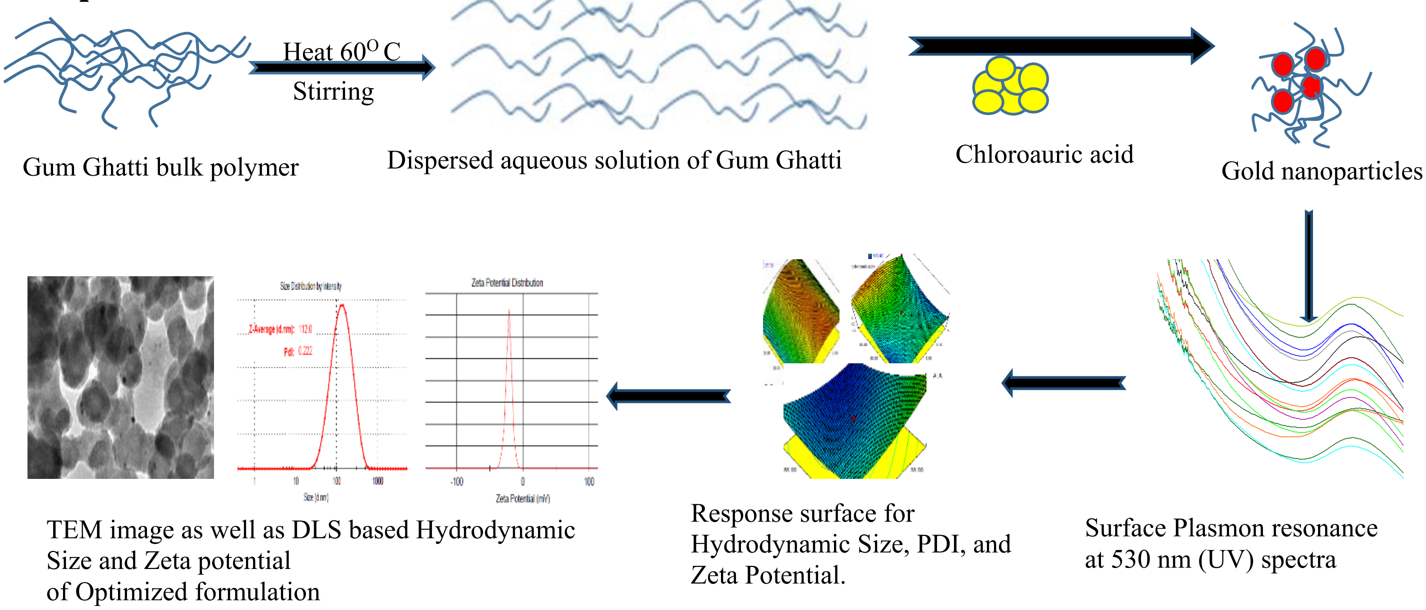
Graphical Abstract



Highlights

- Role of Gum ghatti as a reducing agent, capping agent as well as stabilizing agent for the green synthesis of Gold nanoparticles was investigated.
- During the facile synthesis, toxic reagents or maintaining alkaline pH range not required.
- Impacts of thermal energy (temperature) as well as volume of Gum and Gold solution on size, PDI and zeta potential were analyzed using 3^3 Box-Behnken model.
- On particle size, thermal energy adversely affected the desired smaller sized contributed by the gum and gold solution.
- Zeta potential value increased with both high thermal energy and gum solution while with gold solution it’s being reduced.

- Uniform distribution of these particles was favored by both high temperature and gum solution while adversely impact was seen with the gold solution.

Abstract

Due to unique inherent catalytic characteristics of different size, shape and surface functionalized Gold nanoparticles, their potential application is being explored in various fields such as drug delivery, biosensor, diagnosis and theranostics. However Conventional process for synthesis of these metallic nanoparticles utilizes toxic reagents as reducing agents, additional capping agent for stability as well as surface functionalization for drug delivery purposes. Hence, in this work suitability of Gum Ghatti for reducing, capping and surface functionalization during the synthesis of stable Gold nanoparticles were duly explored. Role and impact of key process variables i.e. volume of chloroauric acid solution, Gum solution and temperature at their respective three different levels, as well as mechanism of formation optimized Gold nanoparticles were also investigated using Box- Behnken design. These novel synthesized Optimized Gold nanoparticles were further characterized by UV spectrophotometer for its SPR at around ~530 nm, DLS for its hydrodynamic size (112.5 nm), PDI (0.222) and Zeta potential (-21.3 mV) while Transmission electron microscopy further revealed surface geometry of these nanoparticles being spherical in shape.

1. Introduction

Adaptations to nanotechnology approaches have emerged as a great tool to equip bulk molecules with an aim to incorporate some novel dimensions into the final processed product. Such type of modulation of the material at nanoscale level often produce product with enhanced potential as well as encourage the researcher across the different disciplines to further explore its mechanism, efficiency and various characteristics in a way so that systemic incorporation of generated information would help to provide not only its wide applications but also simple and reliable solutions to many of pre-existing scientific issues and challenges either with any product or process (1). However, conventional chemical reagents for synthesis of metallic nanoparticles possess strict regulatory concern due to issues like biocompatibility and toxicity, contributed there by the chemical agents used to synthesize them (2). Hence, nowadays considerable attention is growing to utilize principle of green chemistry to synthesize metallic nanoparticles, especially Gold nanoparticles, using abundance, cheap, biodegradable materials sourced from biogenic route such as microorganism as well as extracts of various plant parts including fruit, peel, bark, roots, leaf for their potential role as reducing agents (3). However, many of these processes require additional capping agents to provide considerable stability in the product during their storage as well as after the in-vivo dosing. In the past, many natural components have been investigated as reducing agents but many of them are unstable in nature, hence such metallic nanoparticles required some stabilizing or capping agents during the synthesis and storage (4). Some recent work on plant-based exudate gums and polymers suggest that few natural and semi-synthetic derivatives of these Gums such as gum Karaya, gum Xanthan, gum Gellan, gum Acacia and gum Kondagogu could be a better alternative to synthetic reducing agents, so these types of natural occurring materials were established as good stabilizing agents during the biosynthesis of Gold nanoparticles but the presence of suspending insoluble constituents of these gums, imparts stability issue as a serious challenge (5).

Adaptation of traditional methodologies usually reveals the effects and impacts of only one variable at a time. Although from the statistical point of view such approaches, at the preliminary level of the investigation, seem to be easier to the investigator for manipulating the process but the absence of control over the process limits our understanding and control of the process. Furthermore, previous literature considered nature of process affecting factors as an independent effect while these factors may also exhibit some interdependent effects i.e., the involvement of two or more factors in terms of their interactions. Hence, an adaptation of Box-Behnken, a fractional factorial design, analyzes the both independent as well as interdependent interaction effects of influencing key factors, at their different levels, such effects were further validated using analysis of developed model with different types of statistical test. While analysis of process performance using traditional way have always been found to be insufficient and misleading in nature because its controls over the process are inadequate in nature. Furthermore, prediction of the final outcome, as well as process optimization through traditional way including OFAT (one factor at a time), exhibits a lot of experimental errors which would be possible also due to facts that these variables are confounding in nature. Hence, The factorial design not only bears advantage of simplification of the process when coupled with statistical tests but it also leads to better performing, well validated and more robust system, as per QbD methodology. Additionally, factoring design also enables statisticians and researchers to develop a performance mimicking mathematical model which may help to manipulate effects of single isolated variable, to predict the final outcome at different levels of these factors as well as to grid search composition of these variables for our customized and desired performance. However, only meticulously planned, and statistical control based design may able to yield a reliable model with minimized error to achieve the aim and objective functions of optimization (https://explorable.com/factorial-design).

Gum Ghatti (Indian Gum, CAS number 9000-28-6), name Ghatti originated from mountain passes (ghats), is a high molecular weight (8.94×10^7 g/mol), natural occurring, bland tasted, odourless, water-soluble, amorphous, translucent, non-starchy arabinogalactan type polysaccharide exudate ; obtained from *Anogeissus latifolia* tree of the Combretaceae family (6,7). It is processed by removal of bark, sand and silica followed by sifting, aspiration, and density-table separation. It is composed of L-arabinose, D-galactose, D-mannose, D-xylose and D-glucuronic acid with their molar ratio of 10:6:2:1:2 along with traces of 1% of 6-deoxyhexose (8,9). It occurs naturally as mixed complex of calcium, magnesium, potassium and sodium salt (7). Gum Ghatti as a food ingredient (USFDA Code 184.1333) and falls under "generally recognized as safe" (GRAS); emulsifier and emulsifier salt category (10). It is also approved as food grade additive by Bureau of Indian Standards, India as well as, from a longtime gum ghatti is being used in Japan, India, China, South Korea, Singapore, Russia, Australia, South Africa, Iran, Saudi Arabia, Latin America etc., as approved materials in the preparation of hydrocolloids, butter, beverages, syrup, tablets, flavor fixative, polymerization, emulsion for lipid and waxes (11). It possesses excellent emulsifying & stabilizing characteristics as well as exhibits non-

Newtonian behavior (7). It is also being used for creating water protective layer at acidic pH 4.8 because of exhibiting alkali mediated viscous solution even at 5% concentration (7,11).

Hydrocolloid of Gum Ghatti contains up to 80% soluble dietary fiber and its matrix is enzymatically degraded by some specific gastrointestinal microflora as *Bifidobacterium longum*, so exhibits excellent prebiotic function by sustaining many types of useful bacterial flora on its matrix in the colon, for a very long time (12). It is very effective in the treatment of diarrhea, hypolipidemia and diabetes (13). In Indian system of traditional medicine, because of such properties, it is well established as a rich source of nutrients to the breastfeeding mothers and dependent child, as well is also believed to relieve from a backache, common in post-delivery mothers (12,14).

In this perspective, because of properties of Gum Ghatti (Anogeissus latifolia); a natural origin source of carbohydrate with good abundance and low cost, biodegradable and nontoxic, intermediate viscosity, superior emulsifying properties, relatively higher stability against pH and electrolytes (potential to destabilize the Gold nanoparticles) as well as exceptional interfacial characteristics with faster kinetics; role of Gum Ghatti as reducing, capping and stabilizing agent as well as its mechanism and impact along with other process variables, in one pot, facile and green synthesis route (non-hazardous agent) for development of Gold nanoparticles; by reduction of chloroauric acid; was thoroughly explored through Box- Behnken based design and incorporating other statistical tools.

2. Materials and methods

2.1. Materials

Gum Ghatti was purchased from S.D Fine (India) and Tetrachloroauric acid ($HAuCl_4$) were purchased from Sigma–Aldrich (St Louis, MO, USA). All the Chemicals and water used were of analytical grade.

2.2. Preliminary formulation study

Preliminary formulation studies were also being carried out by using gum Karaya, gum Xanthan, gum Tragacanth, gum Arabica. However, due to inherent challenges such as partial solubility as well as high viscosity, poor reproducibility and further validation of those formulations made them unfit for the formulation development.

2.3. Preparation of Gum solution

The aqueous polymeric solution of Gum was prepared by dissolving 1 g of the Gum into 100 ml water followed by heating at 40 ^oC for overnight under continuous stirring followed by filtration of solution to prepare water soluble lyophilized powder, to be used for preparation of gum solution (1% w/v) during the synthesis steps.

2.4. Synthesis of Gold nanoparticles

On the basis of the Box-Behnken design matrix, the sample gum solution (1% w/v) preheated at desired temperature, under constant stirring, required volume of $HAuCl_4$ (0.1 M) was added in dropwise manner which led to changing color of solution from yellow to purple - ruby red color colloidal solution (characteristic of newly synthesized Gold nanoparticles). The prepared formulation was centrifuged at 20,000 rpm for thirty minutes followed by collection of gold nanoparticles and dilution with double distillate water to be its immediate storage at 4^oC temperature under dark condition before characterization.

2.5. Optimization studies by Box--Behnken Design using RSM

The application of nanomaterials always depends upon the size and stability of the nanomaterial synthesized. Earlier, One-factor-at-time (OFAT) were being used in which at one time only one factor was varied to identify the influence of each factor towards the characteristics of final formulation (15). However, these types of approach have their own limitation because of lack of information about interaction effects of these factors at various levels (16). Hence, in this work RSM approach were adopted using Design expert software (version 7.0.0) to analyze 17 runs (12 main design point and 5 central points) which were generated by the Box-Behnken design with Gum solutions (2 - 4 ml), temperature (70 - 90 ºC) and Gold solution (20 - 40 µl) as independent variables while, particle size, PDI and zeta potential were kept as dependent variables in this experiment. To understand individual and interaction effects of selected variables on the initial performance of reaction in terms of particle size, PDI and zeta potential, response surface methodology was adopted which established a mathematical relationship and coefficient of second order polynomials equation. Linear, quadratic and cross-product term effects of the above variables were used to define second order polynomial equation as a mathematical model for the responses. However, because the third order terms are fully aliased with linear effects (e.g. $X^3 = X$), Box- Behnken model does not consider any of third order terms, While, to achieve a better fit during the lack of fit experiments, effects of $X_1X_2^2$ and $X_1^2X_3$as second order terms were incorporated into this model (17).

$$Y = \beta_0 + \sum_{\iota=1}^{K}(\beta_\iota X_\iota) + \sum_{\iota=1}^{K}\left(\beta_{\iota j} X_\iota^2\right) + \sum_{\iota<j}^{K} \sum_{k=0}^{K} \beta_{\iota j} X_\iota X_j + \mathcal{E}$$

$$X_\iota = \frac{X_{\iota -} X_0}{\Delta X_\iota}$$

Where, Y refers to response of nanoparticles (size and charges, independently), k the number of factors (3 here) in the experiment, β is regression coefficient, Ɛ is residual error, X_ι is real value of independent variables (dimensionless) X_0 is central point value of independent variables while ΔX_i is steep change value. Probable multicollinearity effects due to correlation of third order terms with linear effects are being avoided on the basis of variance inflation factor (VIF) as an acceptance criterion for regression coefficients (18). On the basis of knowledge acquired from initial screening trials of process variables and its three levels [−1, 0, +1], the effects on size and potential of nanoparticles were further statistically analyzed using Design Expert 7.0. ANOVA tests were used for analyzing the amount of variance contributed in a given sample by different factors i.e. for balanced data (equal numbers of observations for each level of a factor) and for unbalanced data, there are different ways to calculate the sums of squares which yield the influence of particular factors (including interactions) which can be estimated through differences between different models. The numerator of the general linear F-statistic, denoted as SSE(R)-SSE(F) i.e., “sequential sum of squares" or "extra sum of squares” approach is one of the approach which is based on principle of weighted marginal means, so the outcome is dependent on the realized sample sizes, namely the proportions in the particular data set or in other terms, testing of first factor without controlling the other (18,19). In this approach either the error sum of squares (SSE) are reduced or there are increase in the regression sum of squares (SSR) with the addition of one or more predictor variables to the model. In short, when a predictor is added to a model, there are some explainable variability in the response, as well as reduction in some of the error value, so it quantitatively analyses the amount of variability (increase in regression sum of squares) or alternatively extent of error reduced there i.e., reduction in the error sum of squares (20).

2.6. Characterizations of Gold nanoparticles

2.6.1. Surface Plasmon resonance (SPR)

Surface Plasmon resonance of formulation were analyzed using U.V -1600 which is a Double Beam UV-Visible Spectrophotometer (Shimadzu corporation, Japan) and spectra were recorded and further proceed by using UV Probe 2.33 software (Shimadzu corporation, Japan).

2.6.2. Hydrodynamic diameter and polydispersity

The hydrodynamic diameter and polydispersity index of formulations were determined in on the principle of dynamic light scattering at 25 ^{o}C with a backscattering angle of 173^{o} using Zetasizer NanoZS (Malvern instrument Ltd., Malvern, UK) after appropriate dilution of formulation so that particle count rate should preferably between 100-300 kcg.

2.6.3. Surface charge (Zeta potential)

For electrokinetic properties (Zeta potential), sample formulation was diluted 10 times using MiliQ water before placing it in disposable zeta cell, followed by measurement which were taken at a temperature of 25^{0}C using Zetasizer Nano-ZS instrument.

2.6.4. Transmission electron microscopy (TEM)

The Transmission Electron Microscopy (TEM) analysis of nanoparticles was performed with a transmission electron microscope operating (Technai- G2, FEI, Holland) at an accelerated voltage of 200 KV.

3. Result and discussion

3.1. Preliminary formulation study

The preliminary formulation studies for the synthesis of gold nanoparticles from water insoluble polymers such as, Gum Karaya and Gum Tragacanth, yielded undesired products in terms of reproducibility, due to their inherent challenges such as, their low solubility as well as stability, contributed by the insoluble fractions of these gum, compromising above 50 % of total polymer weight (which was also responsible for reducing properties of these gums). Hence, optimization of process variables, their control as well as prediction of outcomes of developed final product using any experimental design may also be misleading due to the presence of high amount of bias and error. While other polysaccharides such as Xanthan and Arabica gum, enhances the viscosity, hence mobility and uniform dispersion of gold solution being reduced. The continuous stirring is an important requirement for the synthesis of gold nanoparticles, to prevent the seed-mediated growth of gold nanoparticles as well as their uniform distribution throughout the formulation. However, Gum ghatti, a water soluble and low viscous polymer, inherently devoid of all these challenges even if present at very high concentration.

3.2. Surface Plasmon resonance

Figure 1 above shows the various UV–VIS absorption spectra of Gum Ghatti stabilized Gold nanoparticles synthesized at different temperature levels using the different volume of Gum and Gold solution. These spectra exhibited a characteristic absorption band of Au nanoparticles around 530 nm owing to the Surface Plasmon Resonance (500-600 nm) of these colloids which confirmed the synthesis of colloidal Gold particles during the reaction. However, depending upon size, shape, distribution as well as the composition of the analyzed mixture it was inferred that there is slight shifting in some of the sharp peaks (Fig: 1). Hence UV–VIS absorption spectra shouldn't be taken as quantitative tools rather it should be restricted as a qualitative tool only.

3.3. Screening of factors and variables

Previous literature suggested that due to the presence of the suitable chemical group in natural polymers, these polymers prove a better alternative to synthesis of stable Gold nanoparticles.

Such nanoparticles exhibit superior biological performance, higher drug loading as well as better stability as these polymers, also act as capping agents. No systematic and standard approaches have been published earlier, which signify and emphasize on the screening of key factors (as well as main and interaction effects of these individual factors at their different levels in term of the final response of Gold nanoparticles). Hence in this work, various factors were preliminarily screened as probable key process variables by using both hit and trail method as well as simple OFAT (One factor at a time) based experiment design. Because there was no previous literature available either on role and effect of Gum Ghatti in the synthesis of Gold nanoparticles, we initially determined the minimum level of Gum Ghatti which would be sufficient to reduce Gold solution to produce Gold nanoparticles. So we found that below 1% Gum solution, reaction progress was inefficient while above 4 % of Gum solution there was nucleation mediated size growth in some of the formulation which may be attributed to immobility of Gold nanoparticles in the solution under the influence of viscosity. We further screened out three factors volume of Gold solution, temperature and volume of the polymer solution as critical factors i.e., exhibiting maximum influence there, while other process variables such as the volume of formulation, Gold solution volume, stirring time and stirring speed were fixed throughout the experiments. The volume of the Gold solution was also very critical because, above the critical limit due to the limited reducing capacity of polymeric solution, additional Gold solution ions show nucleation mediated growth phenomena hence quickly increasing the size of nanoparticles, so the solution color tends to be blueish-black instead of purple red. Although, in the case of temperature, we found that increasing temperature above $60^{O}C$ gives the best performance with all formulation. But above $80^{O}C$ there are stability issues with the formulation, especially with low polymeric volume, the reason might be attributed to the thermodynamic behavior of nanoparticles which are being highly sensitive to high temperature or another reason may be given that reducing the potential of polymeric solution was getting affected at a higher temperature. The levels of each of these variables, requirement of Box- Behnken model, under which the combination of factors would yield the Gold nanoparticles during the experiments, are presented in Table 1.

3.4. Formulation optimization using Box-Behnken design

The traditional approach for optimization in the previously published literature were carried out by different reducing agents as well as natural polymers using OFAT, in which one parameter is changed while others are kept constant, and monitoring is done throughout the experiment. However, such conventional technique does not estimate the interactive effects among the variables studied. Hence the actual influence of individual parameter on the response can never be analyzed. Besides, such type of experiment rather focuses on any one or two factors, while the number of experiments under such approach also increases with the increasing factors, which needs more time and money. While, advantages of appropriately selected factorial type experimental design not only include the validity, reliability and replicability of experiments but it also helps to predict the final outcome using appropriate statistical methodology and with a fixed level of sensitivity. Box- Behnken (BBD) as a reduced factorial model is second order rotatable experimental design and most desired model for the economy and precise prediction of final outcome. In this rotatable design, each factor is varied at its three different levels and their response as a factor of the main effect is statistically analyzed along with response surface methodologies. In this model mathematical relationship as well as the coefficient of second order polynomial equations enhance better understanding of individual and interaction effects of selected variables on the initial performance of reaction in term of response, such as Hydrodynamic size, PDI and Zeta potential.

So, here in this study, Box-Behnken design was employed on the basis of knowledge acquired from initial screening trials of process variables and its three levels [−1, 0, +1], the effect of independent variables, i.e. volume of Gold solution, the volume of Gum solution and temperature of the reaction. Hence, a total number of 17 experimental runs were performed and their response was statistically analyzed using Design Expert 7.0 to determine their influence on hydrodynamic size, PDI and potential of nanoparticles in term of response as shown in Table 1.

3.5. Model fitting and statistical analysis

The quality of the data obtained in the polynomial model was expressed by the coefficient of determination (R), the value of adjusted R of the model, Fisher ratio value (F) and standard error of the estimate (SE). The significance of each term in the equation was to estimate the goodness of fit in each case. The analysis of variance tables were generated and the regression coefficients of individual linear, quadratic and interaction terms were determined. The P values of less than 0.05 were considered to be statistically significant throughout the study. While relatively larger Fisher-ratio value signify that most of the variation can be explained by components of any regression equation, not by noise (Zunlai et al., 2012). The regression coefficients were then used to make statistical calculations to generate contour and dimensional maps from the regression models. Probable multicollinearity effects due to correlation of third order terms (Such as A^3, B^3 and C^3) with linear effects (A, B and C respectively) are being avoided on the basis of variance inflation factor (VIF) as an acceptance criterion (for simplification, $1^3 = 1$ and $-1^3 = -1$) in the regression coefficients.

Although, the highest order term which could also be a good fit was predicted here as Cubic model which was estimated as aliased model. However, this was being ignored because model terms estimated here in this design was too low to predict fit of the cubic model. Hence, the

Quadratic model was selected as highest models w.r.t., linear and 2FI models as best fit for all three responses being analyzed there i.e., Hydrodynamic Size, PDI and Zeta potential.

Here, in the case of sequential model; sum of square test, larger Fisher-ratio value (83.37, 70.55 and 78.34) as well as their low P value (<0.0001) for Hydrodynamic size, PDI and Zeta potential respectively for the Quadratic model. This strongly indicated statistical significance of Quadratic model as highest order polynomial where additional terms are significant as well as the model is not biased. Hence Quadratic model defines regression equation very well (Table 2). However, any regression model may further fail to adequately describe the functional relationship between the experimental factors and the response because of missing of important terms from the model such as interactions or quadratic terms or there are unusually large residuals when fitting the model, hence lack of fit test was being carried out which revealed that the selected quadratic model has desired relatively insignificant characteristics. Here, in the case of Lack of fit test, low Fisher-ratio value (1.82, 3.16 and 4.04) as well as High P value (0.2838, 0.1480 and 0.1053) for Hydrodynamic size, PDI and Zeta potential respectively for Quadratic model, further suggested that model also exhibits moderately fit to the model (Table 3). While, further, to prevent the chance of over-fitting a mathematical relationship was established between the predictor and response variables for the data set used in the model calculation PRESS (prediction sum of squares) values (223.72, 8155.13 and 58.58) were calculated (smaller are desired) for Hydrodynamic size, PDI and Zeta potential respectively, as a mean to provide valid predictions for new observations i.e. model predictive ability (Table 3). Similarly, In the Model summary statistics of Hydrodynamic size, PDI and Zeta potential, in which observations were expressed in term of R^2 values (0.98885, 0.9788 and 0.9811) as well as using PRESS data. Adjusted R^2 v/s Predicted R^2 (0.9737 v/s 0.8860, 0.9515 v/s 0.7514 and 0.9568 v/s 0.7655) for Hydrodynamic size, PDI and Zeta potential respectively, were also calculated using those observations which were not included in the model estimation so such analysis became a more intuitive way to interpret the results (Table 3). So, to summarize, the statistical study suggested a quadratic model by supporting sequential model sum of square, lack of fit and model summary statistics. Hence, in another term, we may say that Quadratic model as the best model to establish a relationship between the independent variables as well as to predict the response (Table 3).

The model adequacy was further checked using ANOVA (Table 4). Analysis of variance table for response surface reduced quadratic model (Partial sum of square III) implied that model was highly significant based on its low P value < 0.0001, which suggest that there are less than 0.01 % chance that a model of such large F value (68.01,49.39 and 29.76) for Hydrodynamic size, PDI and Zeta potential respectively, could be sourced from noise. The model terms A, B, C, AB, AC, BC, A^2, C^2 were significant in case of Hydrodynamic size, while A, B, C, BC, A^2, B^2, C^2 in case of PDI, similarly model terms A, B, C, BC, A^2, C^2 were found to be significant with zeta potential. Hence, the model was reduced by removing the insignificant interaction and square terms from the equation. Reducing the model terms improved the fit parameters studied earlier, such as various lack of fit parameters which were signified by comparing lower F value (1.97 v/s 1.82 for Hydrodynamic size and 6.89 v/s 4.04 for Zeta potential) and higher P value (0. 2062 v/s 0.1480 for PDI). Similarly; values of predicted R^2 which are considered as estimators of better fit of data and its prediction, also improved (0.9021 v/s 0.8860 for Hydrodynamic size, 0.8910 v/s 0.7514 for PDI while 0.8179 v/s 0.7655 for zeta potential). Consequently, the adequate precision value (above 4 are desired) which signify noise to signal ratio was found to be 30.967 for Hydrodynamic size,21.146 for PDI and 17.937 for Zeta potential, which demonstrated that there was an adequate signal available to navigate the design space which gave final polynomial equation as below.

3.6. Final Equation in Terms of Coded Factors

R 1 = 123.46 - 2.24*A - 4.39*B + 9.86*C + 3.73*A*B - 4.51*A*C - 2.34*B*C - 9.58*A^2 +10.43*C^2

Hydrodynamic size (nm) = 123.46 -2.24*Gum volume - 4.39*Gold volume + 9.86*Temprature + 3.73 *Gum volume *Gold volume - 4.51 *Gum volume* Temprature- 2.34* Gold volume* Temprature - 9.58*Gum volume^2+10.43*temprature^2

R2 (x 1/1000) = 171.20 -23.25*A + 4.25* B - 23.75* C - 22.00* B*C + 65.15*A^2+14.15*B^2+16.65*C^2

PDI (x 1/1000) = 171.20 -23.25*Gum volume+4.25*Gold volume-23.75*Temprature - 22.00 *Gold volume*Temprature + 65.15*Gum volume^2+14.15*Gold volume^2+16.65*Temprature^2

R3 = -17.61 +1.63*A -0.38* B +2.08* C +2.40* B*C -5.44*A^2-2.44* C^2

Zeta Potential = -17.61 +1.63*Gum volume -0.38* Gold volume +2.08* Temprature+2.40* Gold volume*Temprature -5.44*Gum volume^2 - 2.44*Temprature^2

3.7. Final Equation in Terms of Actual Factors

R1 = 514.66822 +80.17638*A +0.30975*B -13.64026*C +0.37300*A*B -0.45125*A*C - 0.023350*B*C -9.58461*A^2 +0.10425*C^2

Hydrodynamic size (nm) = 514.66822 +80.17638*Gum volume +0.30975* Gold volume - 13.64026*Temprature +0.37300*Gum volume*Gold volume -0.45125*Gum volume*Temprature -0.023350*Gold volume*Temprature -9.58461*Gum volume^2 +0.10425*Temprature^2

R2 (x 1/1000) = 1669.50000 -414.15000*A +9.53500*B -22.41500*C -0.22000*B*C +65.15000*A^2 +0.14150*B^2 +0.16650*C^2

PDI (x 1/1000) = 1669.50000 -414.15000*Gum volume +9.53500*Gold volume - 22.41500*Temprature -0.22000*Gold volume*Temprature+65.15000*Gum volume^2 + 0.14150*Gold volume^2 +0.16650*Temprature ^{2}

R3= -185.43763 +34.23941*A-1.95562*B +3.38984*C +0.023975*B*C -5.43553* A^2 - 0.024380* C^2

Zeta potential = -185.43763 +34.23941*Gum volume -1.95562*Gold volume +3.38984*Temprature +0.023975*Gold volume*Temprature -5.43553*Gum volume^2 -0.024380*Temprature^2

The model terms of mathematical expression of polynomial equations for Hydrodynamic size, PDI and zeta potential expressed either in term of positive value (indicating that these effects are directly proportional to the final response i.e., favour them), while some of the model terms were expressed as negative term (inferring that there were inversely proportional to the final response i.e., oppose same) (Table 4).

3.8. Response Surface methodology

These graphs were plotted using Design-Expert 7.0.0 version software to study the effects of parameters and their interactions on average Hydrodynamic size, PDI and Zeta potential of biosynthesized nanoparticles using 3D-response surfaces methodology (Fig: 2).

The Gum volume (A) was found to exhibit a direct relationship with Zeta potential while inversely related to Hydrodynamic size and PDI. It was found that Gum volume reduced the Hydrodynamic size by approximately 200 %, PDI improved as 1/25 times while Zeta potential increased by 150 %. Such phenomena can be well described in a way that Gum Ghatti is strong reducing and stabilizing agent hence as we increased its volume during the reaction, Hydrodynamic size was reduced and further capped by Gum polymer prevented aggregation of particles, thereby PDI also improved drastically. Similarly, the relationship between polymer Gum Ghatti on Zeta potential was also established based on its capping properties and neutral charge there. The results inferred that lower Gum Ghatti volume was relatively more preferred in terms of providing the surface charge. As we started to increase the volume of Gum it started to cover the surface of Gold nanoparticles, followed by a reduction in absolute value of zeta potential. However, if both Gum volume and Gold volume were varied together then there were approximately 3.5 times increase in the Hydrodynamic size, this may be reasoned because increasing the number of Gold nanoparticles may start nucleation ripening effects leading to increased hydrodynamic size of particles. While if Gum volume and temperature both were varied then Hydrodynamic size was reduced by approximately 1/4 times, which means temperature favor the reaction and enhanced the reducing behavior of Gum Ghatti.

3.9. Effects of Gold volume (B) on Hydrodynamic size (R1), PDI (R2) and Zeta potential (R3)

The Gold volume (A) were found to exhibit a relationship which favored the absolute value of Zeta potential as well as desired lower Hydrodynamic size and PDI. It was found that Gold volume reduced the Hydrodynamic size and PDI by approximately by 400 %, while Zeta potential improved by 38 %. The role of Gold volume to lower Hydrodynamic size may be described in a way that as we increased the Gold volume, more Gold nanoparticles were generated followed by attachment of unused Gum Ghatti polymers to its surface hence polymer aggregation was avoided and also the PDI too improved. While as far as Zeta potential was concerned, it is already established that after reaching a critical point, if more capping agents were attached then the absolute value of zeta potential also lowered, hence if the Gold volume was increased then it utilizes most of the unused polymers at the individual surface, so preventing reaching of critical points. However, if both Gold volume and Temperature were varied together then there were approximately 250 % reduction in the Hydrodynamic size and absolute value of Zeta potential as well as 22 times reduction in PDI which can be further ascertained in a way that with increasing the Gold volume if higher temperature were used then it provides the better reducing behavior hence preferred, however, such higher temperature also affect the Zeta potential which may be attributed to temperature mediated degradation of Gum polymer .

3.10. Effects of Temperature (C) on Hydrodynamic size (R1), PDI (R2) and Zeta potential (R3)

The temperature (C) was found to exhibit an inverse relationship with the absolute value of Zeta potential by 200%, simultaneously increasing the Hydrodynamic size by 10 times while PDI favored by reducing 25 times, approximately. The effects of temperature at higher temperature may be attributed as it reduces the PDI value i.e., distribution range of Hydrodynamic size become narrow and more uniform however the temperature could increase the kinetic energy, thereby Hydrodynamic size of particle was increased due to collision mediated nucleation effects, while it also reduces the absolute value of zeta potential too. However, when both Gum volume and Temperature were varied together then temperature aids to provide a better reducing environment to Gum Ghatti. Hence Hydrodynamic size was reduced by approximately 500 %, while when Temperature and Gold volume both were varied together then there was – 22 times reduction in PDI, hence favoring the narrow and uniform distribution size range, although such higher temperature also disturbed the zeta potential absolute value by half.

4. Optimization of influencing factors and model validation

Optimizations of influencing factors were aimed mainly to determine the optimum value of variables that yield the highest absolute value of zeta potential, lowest hydrodynamic size and PDI. Numerical optimization was performed using Design Expert V.7.0.0. Hence, to validate the design model which were set to obtained Gold nanoparticles with the goal to minimum Hydrodynamic size and PDI but maximum absolute Zeta potential value, a point prediction based analysis was carried out with the given parameters to compare the final product performance with the theoretical one. For Validation of optimized study, three confirmatory runs were performed as a tool for very high degree of prognostic ability of mathematical methodology. It was found that the design predicted optimized formulation at Gum volume 4 mL, Gold volume 30.76 µL and Temperature 76.72 ^{o}C which predicted Gold nanoparticles with hydrodynamic size of 111 nm (CI range : 108.52-113.49 nm) and its PDI 0.222 while the mean experimental value for hydrodynamic size was found to be 112.5 nm and its PDI 0.222 similarly, predicted zeta potential value was -22.38 [CI range: (-23.78) – (-20.970)] while average experimental zeta potential was found -21.3, which were in close accordance with the theoretical statistical value, hence verify that the model is satisfactory in predicting the response so may be concluded that response surface methodology is a reliable tool to optimize the formulation.

Conclusion

Here in this work, we investigated the role of Gum Ghatti as reducing agent in the synthesis of Gold nanoparticles by green chemistry approach. After the identification of key process factors (independent variables) through conventional experiments, we identify the Gum volume (mL), Gold volume (µL) and Temperature (^{o} C) as probable factors, hence Box- Behnken design were applied to identify role of gum, Gold, and temperature, as well to optimize the final product. Multivariate experimental design predicted a quadratic model as the functional relationship between Hydrodynamic size, PDI and Zeta potential with these three independent variables, expressed as Response surface methodology. A satisfactory goodness-of-fit was also observed between the predictive and experimental results.

It was found that increasing gum concentration reduced the hydrodynamic size of the gold nanoparticle by more than 200% while, increasing gold nanoparticles reduces the size of nanoparticles lower than half with respect to effects of gum solution. However, the temperature was found to be critical factor which affects the stability adversely and promote size growth by approximately 10 times. Thus rather than gum and gold concentration, the prime concern should be on the controlling the temperature of reaction during the preparation of gold nanoparticles, although as described above while, raising the temperature, faster the reaction rate too. Although as far as PDI was concerned, increasing gold volume there favor broad distribution of nanoparticles while the presence of gum ghatti was found to be critical for controlling both size and its distribution there in the final solution. Here, overall temperature effects were found to produce larger size nanoparticle perhaps due to seeding mediate growth hence, the distribution would be narrowly in a manner as the model further suggested here.

The advantage of Box-Behnken model was to establish a mathematical relationship with critical process variables with the product final characteristics. Such, model not only advance our understanding of the role of different factors with individual parameters of performance in a quantitative manner but also it provides several possible combinations of required level of each factor which produce particles with any desired performance. It also predicts the desired performance of each characteristic if the combination of factors were varied there. The optimized Gold nanoparticles of spherical size were further confirmed by TEM analysis. Using point prediction analysis which emphasis on desired attributes required in the final formulation, optimized nanoparticles were synthesized and its mean hydrodynamic size, PDI and Zeta potential were found to be 112.5 nm, 0.222 and -21.3 mV, respectively.

5. Acknowledgements

Authors gratefully acknowledge instruments support receive from the K.R Mangalam University, JMI (New Delhi) and AIIMS (New Delhi) India, during these experiments.

References

1. Jayakumar, R., Menon, D., Manzoor, K., Nair, S. V., & Tamura, H. (2010). Biomedical applications of chitin and chitosan based nanomaterials—A short review. Carbohydrate Polymers, 82(2), 227-232.
2. Yan, W., Chen, C., Wang, L., Zhang, D., Li, A. J., Yao, Z., & Shi, L. Y. (2016). Facile and green synthesis of cellulose nanocrystal-supported gold nanoparticles with superior catalytic activity. Carbohydrate polymers, 140, 66-73.
3. Kora, A. J., Sashidhar, R. B., & Arunachalam, J. (2010). Gum kondagogu (Cochlospermum gossypium): a template for the green synthesis and stabilization of silver nanoparticles with antibacterial application. Carbohydrate Polymers, 82(3), 670-679.
4. Yan, J. K., Liu, J. L., Sun, Y. J., Tang, S., Mo, Z. Y., & Liu, Y. S. (2015). Green synthesis of biocompatible carboxylic curdlan-capped gold nanoparticles and its interaction with protein. Carbohydrate polymers, 117, 771-777.
5. Pooja, D., Panyaram, S., Kulhari, H., Rachamalla, S. S., & Sistla, R. (2014). Xanthan gum stabilized gold nanoparticles: characterization, biocompatibility, stability and cytotoxicity. Carbohydrate polymers, 110, 1-9.
6. Ramawat, K. G., Mérillon, J. M., & Ahuja, M. R. (Eds.). (2014). Tree biotechnology. CRC Press.
7. Kaur, L., Singh, J., & Singh, H. (2009). Characterization of gum ghatti (Anogeissus latifolia): a structural and rheological approach. Journal of food science, 74(6), E328-E332.

8. Deshmukh, A. S., Setty, C. M., Badiger, A. M., & Muralikrishna, K. S. (2012). Gum ghatti: A promising polysaccharide for pharmaceutical applications. Carbohydrate polymers, 87(2), 980-986.
9. Wüstenberg, T. (2014). Cellulose and cellulose derivatives in the food industry: fundamentals and applications. John Wiley & Sons.
10. Rahman, M. S. (Ed.). (2007). Handbook of food preservation. CRC press.
11. Kora, A. J., & Rastogi, L. (2015). Green synthesis of palladium nanoparticles using gum ghatti (Anogeissus latifolia) and its application as an antioxidant and catalyst. Arabian Journal of Chemistry.
12. Kora, A. J., Beedu, S. R., & Jayaraman, A. (2012). Size-controlled green synthesis of silver nanoparticles mediated by gum ghatti (Anogeissus latifolia) and its biological activity. Organic and medicinal chemistry letters, 2(1), 1.
13. Parvathi, K. M. M., Ramesh, C. K., Krishna, V., Paramesha, M., & Kuppast, I. J. (2009). Hypolipidemic activity of gum ghatti of Anogeissus latifolia. Pharmacognosy magazine, 5(19), 11.
14. Banavara, D., Alvey, J., & Gonzalez, J. (2013). U.S. Patent Application No. 13/794,270.
15. Kanmani, P., Karthik, S., Aravind, J., & Kumaresan, K. (2012). The use of response surface methodology as a statistical tool for media optimization in lipase production from the dairy effluent isolate Fusarium solani. ISRN biotechnology, 2013.
16. Khosravian, S., Montazer, M., Malek, R. M., & Harifi, T. (2015). In situ synthesis of nano ZnO on starch sized cotton introducing nano photo active fabric optimized with response surface methodology. Carbohydrate polymers, 132, 126-133.

17. Lewis, G. A., Mathieu, D., & Phan-Tan-Luu, R. (1998). Pharmaceutical experimental design. CRC Press.
18. https://onlinecourses.science.psu.edu/stat501/node/347, accessed on 01, May 2017.
19. https://onlinecourses.science.psu.edu/stat501/node/295 accessed on 01, May 2017.
20. http://blog.minitab.com/blog/adventures-in-statistics/multiple-regession-analysis-use-adjusted-r-squared-and-predicted-r-squared-to-include-the-correct-number-of-variables accessed on 01, May 2017.
21. https://explorable.com/factorial-design, accessed on 01, May 2017.

List of Figures

Figure 1: UV–VIS absorption spectra of various newly synthesized and Gum Ghatti stabilized Gold nanoparticle formulations.

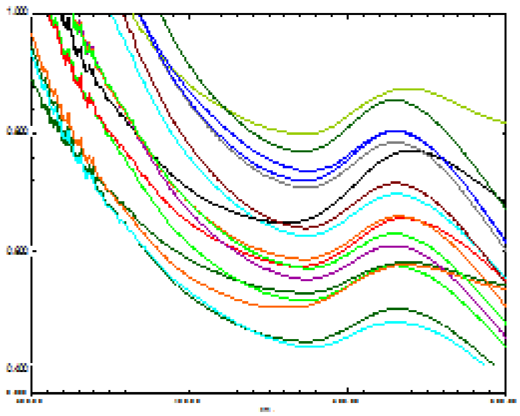


Figure 2 : 3D Response surface graph representing role of various factor on Hydrodynamic size, PDI and Zeta potential.

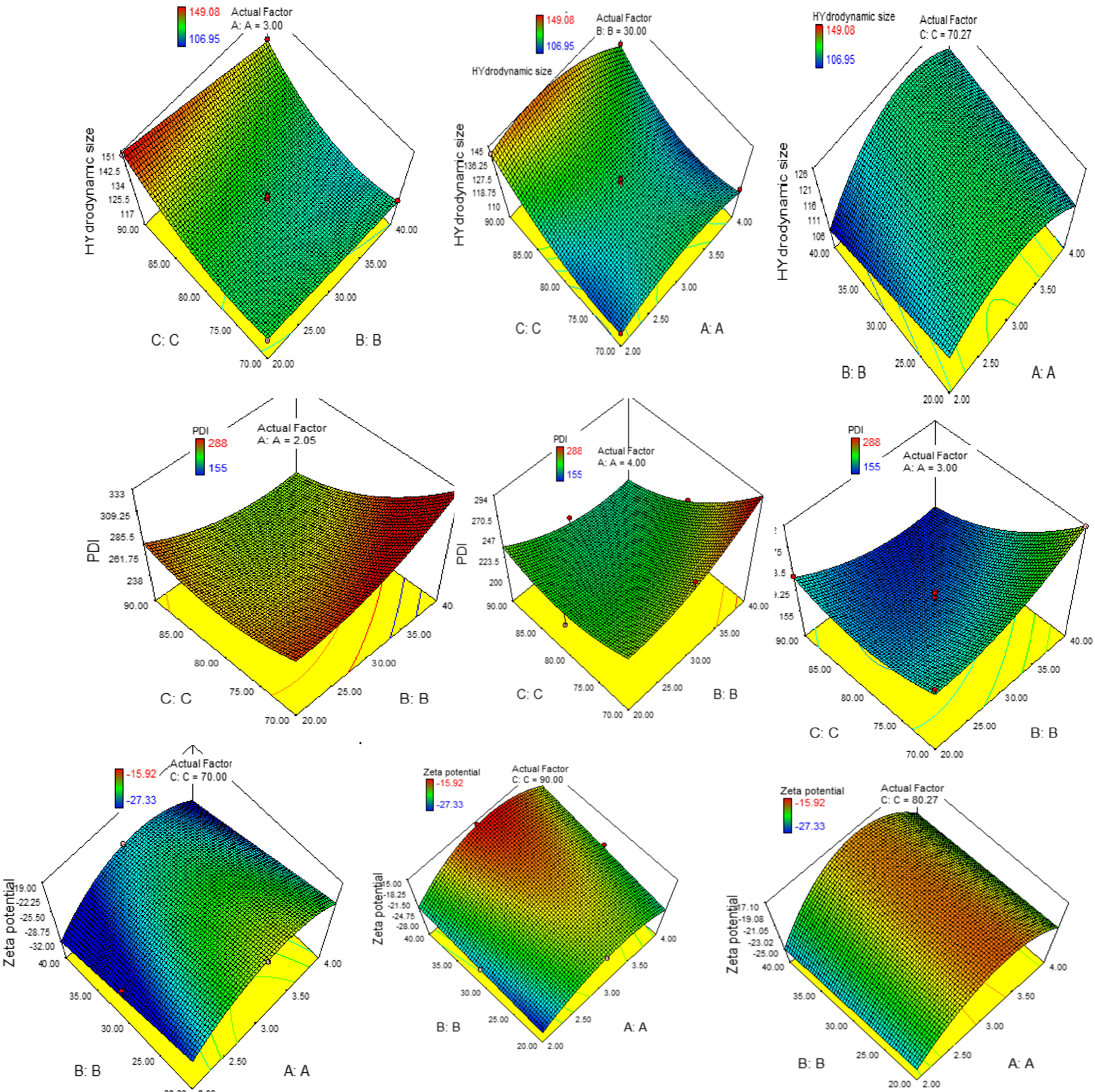
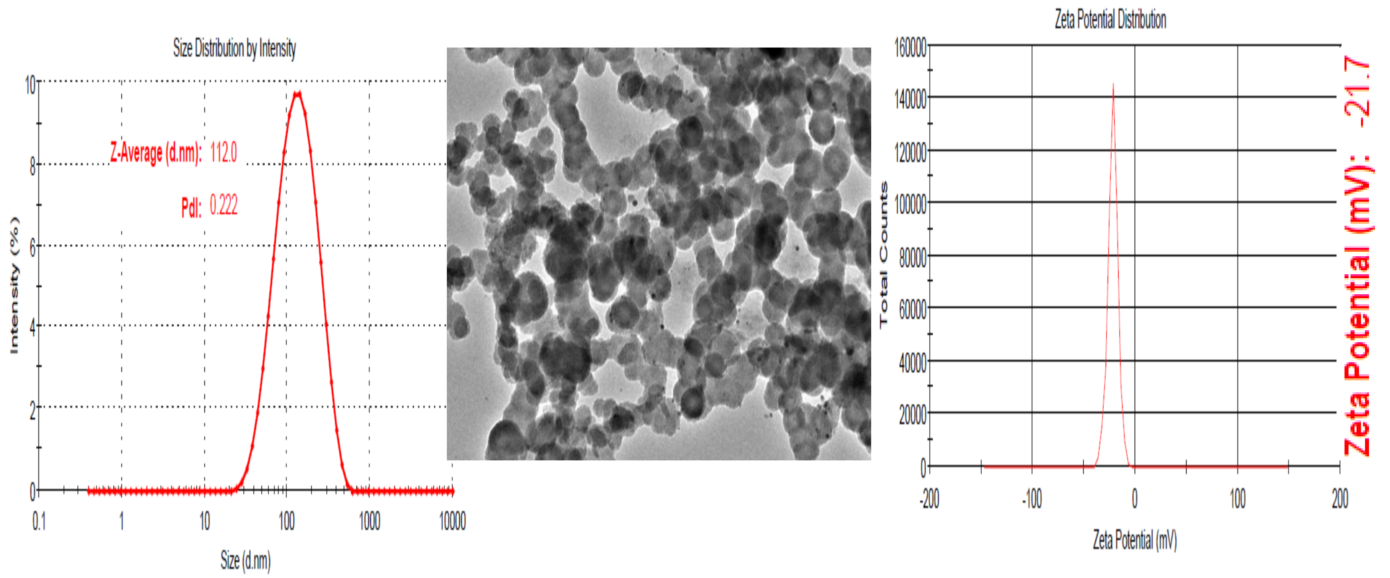


Figure 3: The Hydrodynamic size, PDI, TEM and zeta potential graph of Box-Behnken based optimized formulation.



List of Tables

Table 1 : Impact of three factors, at their three different levels, on final performance as per the Box—Behnken design.

Run	Gum Volume (mL)	Gold volume (μL)	Temperature (ºC)	Hydrodynamic size (nm)	PDI	Zeta potential (mV)
1	3	30	80	149.08±2.65	0.166±0.084	-16.51±2.97
2	3	40	70	124.11±2.43	0.283±0.887	-25.42±4.87
3	3	40	90	125.33±2.02	0.162±0.093	-17.17±5.77
4	2	30	90	112.11±1.76	0.177±0.012	-17.92±3.91
5	3	30	80	123.51±1.14	0.288±0.098	-27.33±4.52
6	3	30	80	121.84±3.68	0.172±0.018	-17.42±2.58
7	2	20	80	128.38±3.33	0.208±0.033	-19.58±3.83
8	4	20	80	118.26±4.13	0.251±0.019	-25.08±2.76
9	3	30	80	140.61±2.17	0.212±0.079	-21.08±4.92
10	2	30	70	125.81±2.57	0.251±0.976	-24.92±5.19
11	2	40	80	125.09±2.85	0.155±0.413	-15.92±3.76
12	4	30	90	123.54±1.67	0.233±0.843	-22.83±5.66
13	3	30	80	112.44±1.66	0.261±0.096	-27.08±3.38
14	3	20	70	122.13± 2.78	0.197±0.064	-20.67±3.88
15	4	30	70	108.65 ± 3.13	0.205±0.046	-20.08±2.61
16	4	40	80	106.95±4.04	0.179±0.007	-17.67±5.87
17	3	20	90	137.76±2.87	0.278±0.025	-25.75±5.09

Variables	Type	Name	Unit	Level: Low (-1)	Middle (0)	High (+1)
Variables	Independent	Gum volume	(mL)	2	3	4
		Gold volume	(μL)	20	30	40
		Temperature	(ºC)	70	80	90
	Dependent	Hydrodynamic diameter	(nm)	106.95	128.015	149.08
		P.D.I	-	0.155	0.2215	0.288
		Zeta Potential	(mV)	-15.92	-21.625	-27.33

Table 2 : Model fitting table for hydrodynamic size, PDI and zeta potential

Statistical Test	**Response Name (Unit)**	**Source**	**Sum of squares**	**Degree of freedom**	**Mean square**	***F value***	***p value***	**Remark**
Sequential model fitting	Hydrodynamic size (nm)	Quadratic	808.2	3	269.4	*83.37*	*<0.0001*	**Suggested**
		Cubic	13.05	3	4.35	*1.82*	*0.2838*	Aliased
	PDI (x 1/1000)	Quadratic	21054.08	3	7018.03	*70.55*	*<0.0001*	**Suggested**
		Cubic	489.5	3	163.17	*3.16*	*0.148*	Aliased
	Zeta Potential (mV)	Quadratic	158.35	3	52.78	*78.34*	*<0.0001*	**Suggested**
		Cubic	3.55	3	1.18	*4.04*	*0.1053*	Aliased
Lack of Fit tests	Hydrodynamic size (nm)	Quadratic	13.05	3	4.35	*1.82*	*0.2838*	**Suggested**
	PDI (x 1/1000)	Quadratic	489.5	3	163.17	*3.16*	*0.148*	**Suggested**
	Zeta Potential (mV)	Quadratic	3.55	3	1.18	*4.04*	*0.1053*	**Suggested**
Statistical Test	**Response Name (Unit)**	**Source**	**Std. deviation**	**R2**	**Adj. R2**	***Pred. R2***	***PRESS***	**Remark**
Model Summary Statistics	Hydrodynamic size (nm)	Quadratic	1.8	0.9885	0.9737	*0.886*	*223.72*	Suggested
	PDI (x 1/1000)	Quadratic	9.97	0.9788	0.9515	*0.7514*	*8155.13*	Suggested
	Zeta Potential (mV)	Quadratic	0.82	0.9811	0.9568	*0.7655*	*58.58*	Suggested

Table 3 ANOVA for the response surface quadratic model for Hydrodynamic size, PDI and Zeta potential.

Response Name (unit)	Significant model terms	Source	Sum of squares	Degree of freedom	Mean squares	*F* value	*p* value	Model summary statistics		
								R^2	R^2Adj	R^2 Pred
Hydrodynamic size (nm)	A, B, C, AB, AC, BC, A^2, C^2	Model	1933.78	8	241.72	68.01	< 0.0001	0.9855	0.9710	0.9021
		Residual	28.44	8	3.55					
		Lack of fit	18.86	4	4.72	1.97	0.2637			
		Pure error	9.57	4	2.39					
		Total	1962.22	16						
PDI (X1/1000)	A, B, C, BC, A^2, B^2, C^2	Model	31971.58	7	4567.37	49.39	< 0.0001	0.9746	0..9549	0.891
		Residual	832.3	9	92.48					
		Lack of fit	625.5	5	125.1	2.42	0.2062			
		Pure error	206.8	4	51.7					
		Total	32803.88	16						
Zeta Potential (mV)	A, B, C, BC, A^2, C^2	Model	236.52	6	39.42	29.76	< 0.0001	0.947	0.9151	0.8179
		Residual	13.25	10	1.32					
		Lack of fit	12.08	6	2.01	6.89	0.0414			
		Pure error	1.17	4	0.29					
		Total	249.77	16						

Table 4 Coefficient of regression model and their significance.

Response	Factor	Coef. Est.	Std. error	95% C.I Low	95% C.I High	F -Value	P-value
Hydrodynamic size (nm)	Intercept	123.46	0.75	121.74	125.19	68.01	< 0.0001
	A-A	-2.24	0.67	-3.78	-0.70	11.31	0.0099
	B-B	-4.39	0.67	-5.93	-2.86	43.42	0.0002
	C-C	9.86	0.67	8.32	11.40	218.87	< 0.0001
	AB	3.73	0.94	1.56	5.90	15.66	0.0042
	AC	-4.51	0.94	-6.69	-2.34	22.91	0.0014
	BC	-2.34	0.94	-4.51	-0.16	6.14	0.0383
	A^2	-9.58	0.92	-11.70	-7.47	109.12	< 0.0001
	C^2	10.43	0.92	8.31	12.54	129.11	< 0.0001
PDI (X 1/1000)	Intercept	171.20	4.30	161.47	180.93	49.39	< 0.0001
	A-A	-23.25	3.40	-30.94	-15.56	46.76	< 0.0001
	B-B	4.25	3.40	-3.44	11.94	1.56	0.2428
	C-C	-23.75	3.40	-31.44	-16.06	48.80	< 0.0001
	BC	-22.00	4.81	-32.88	-11.12	20.93	0.0013
	A^2	65.15	4.69	54.55	75.75	193.25	< 0.0001
	B^2	14.15	4.69	3.55	24.75	9.12	0.0145
	C^2	16.65	4.69	6.05	27.25	12.62	0.0062
Zeta Potential (mV)	Intercept	-17.61	0.46	-18.63	-16.60	29.76	< 0.0001
	A-A	1.63	0.41	0.72	2.53	15.97	0.0025
	B-B	-0.38	0.41	-1.28	0.53	0.85	0.3770
	C-C	2.08	0.41	1.18	2.99	26.19	0.0005
	BC	2.40	0.58	1.12	3.68	17.36	0.0019
	A^2	-5.44	0.56	-6.68	-4.19	94.16	< 0.0001
	C^2	-2.44	0.56	-3.69	-1.19	18.94	0.0014