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A B S T R A C T
At a novel pH value of the polymeric solution (6.2), variable chitosan (Cs) and sodium tripolyphosphate (TPP) concentrations and mass ratios were optimized to improve the process yield without undesirable particle flocculation. Prepared formulations were characterized in terms of particle size (PS), zeta potential (ZP) and percentage yield (%yield). Artificial neural networks (ANN) were built up and used to identify the parameters that control nanoparticle (NP) size and yield, in addition to being tested for their ability to predict these two experimental outputs. Using these networks, it was found that TPP concentration has the greatest effect on PS and% yield. The most optimum formulation was characterized by a notable process yield reaching 91.5%, a mean hydrodynamic PS 227 nm, ZP = 24.13 mv and spherical compact morphology. Successful Cs-TPP interaction in NP formation was confirmed by both Fourier transform-infrared spectroscopy (FT-IR) and differential scanning calorimetry (DSC). This study demonstrated the ability of ANN to predict not only PS of the formed particles but also NP% yield. This may have a great impact on Cs-TPP NPs preparation and can be used to customize the required target formulations.

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1. Introduction

Chitosan nanoparticles (Cs NPs) offer numerous advantages qualifying them to be promising drug delivery systems [1]. Chitosan (Cs) is a biodegradable and biocompatible polymer composed of β1 → 4 linked glucosamine and N-acetyl glucosamine monomer and it is manufactured commercially on a large scale by the N-deacetylation of the abundant biopolymer, chitin; isolated from the exoskeleton of crustaceans such as crabs and shrimps [2]. Cs is a hydrophilic polycationic polymer at acidic pH, which in the presence of polyanions such as pentasodium tripolyphosphate (TPP) can spontaneously gel into submicron sized particles. This famous ionotropic gelation method is relatively simple, mild and avoids the use of organic solvents and high temperatures. Since first described by Calvo et al. [3]. Cs ionotropic gelation has been extensively studied en route for obtaining nanocarrier systems with tunable geometries, positive surface charge and a good capacity of drug encapsulation.

However, limited body of literature incorporated results on the production method yield [4], possibly because it is relatively low. Nowadays, Cs-based nanocarriers are under advanced pre-clinical and early clinical development as reported by Garcia et al. [1]. Therefore, enhancement of Cs-TPP NP production yield would, indeed, be important for increasing the productivity of this preparation method when applied on the industrial scale. Moreover, more NP production means more nanovehicles available in the formulation which would reflect on improving drug loading efficiency. In a recent study [5], our team demonstrated the enhancement of yield and diminution of Cs-TPP NPs when both polymer and cross-linker solutions were used at elevated pH conditions and lowest possible mass ratios. Accordingly, in the present work, our team attempts to use a novel Cs solution pH 6.2 to favor the preparation of electropositive Cs-TPP nanosuspension with maximized NP yield.

To our best knowledge, this is the first study that investigates the use of Cs solution at a pH 6.2 for the preparation of Cs-TPP NPs. However, this pH value is considered critical; the reduced charge...
density on polymer chains (pK_a = 6.5) might render the polymer susceptible to aggregation. Accordingly, it was important, in this study, to evaluate the effect of increasing Cs solution pH and concentration on NP productivity and properties, but as well important was to identify the concentration and amount of cross-linker used favoring the preparation of a monodisperse NP population. Cs-TPP NP preparation is a multivariate process; the ultimate characteristics of CsNPs are influenced by the various formulation parameters. In recent years, artificial neural network (ANN) has been introduced in pharmaceutical research as a powerful tool for modeling and analyzing complex multivariate processes [6].

The architecture of ANNs is biologically inspired to simulate the way in which the human brain processes information ANNs are generally presented as machine learning computer programs composed of interconnected processing units called “neurons” which send messages to each other. In most cases identical neurons are interconnected in at least three separate layers; the input data are received in the input layer and the weighted sum of all inputs is computed in the hidden layer(s), with the output results reported through the output layer [7]. The connections have numeric weights that can be tuned based on training experience, making neural nets adaptive and capable of learning. Nowadays, ANN is successfully applied in the analysis and modeling of complex multivariate nonlinear relationships frequently encountered in pharmaceutical research and that are difficult to model using classical statistical methods [8,9]. With the added benefits of short computing time and high potential of quality adaptive performance, these artificial intelligence tools have been successfully used in the design of controlled release delivery systems [10], in the evaluation of in vitro–in vivo correlation for dry powder inhalers [11], also used for improving the understanding of the process of formation of nanoemulsion [6] and the determination of the factors controlling the particle size and entrapment efficiency of noscapine in PEG/PLA nanoparticles [12].

The aim of this study was to characterize Cs-TPP NPs formulated at novel Cs solution pH 6.2, when Cs and TPP concentrations and mass ratios were used as experimental variables by applying ANN in identifying the factors specifically influencing NP yield and size.

2. Materials and methods

2.1. Materials

Low molecular weight (Mw) Cs (75–85% degree of deacetylation, viscosity 20 to 300 cP, average Mw ~ 50 kDa, Cat. No.: 448869) and pentasodium tripolyphosphate (TPP) were purchased from Sigma–Aldrich (St. Louis, MO, USA), Sodium hydroxide (NaOH) was supplied from El-Nasr Pharmaceutical Chemicals (Egypt). Deionized water used throughout the research work was produced using a Milli-Q Gradient A10 System. All chemicals and reagents were used as received without further purification.

2.2. Cs solubility assessment at pH 6.2

The solubility of Cs was assessed primarily at such critical pH 6.2 by visual observation for solution clarity and any aggregates formation [13]. Additionally, a quantitative solubility test was performed according to the method adopted by [14] for the highest examined Cs concentration (0.3% w/v) as follows:

A weighed sample was dissolved in 1% acetic acid and stirred at room temperature till complete dissolution, and then the sample was filtered (0.22 μm membrane filter), dried and weighed. The solubility was determined by the following equation:

\[
\text{Solubility\%} = \frac{\text{weight of soluble part}}{\text{total weight of sample}} \times 100
\]

2.3. Preparation of Cs-TPP NPs

Cs-TPP NPs preparation followed the ionotropic gelation method was first described by Calvo et al. [3]. Low molecular weight Cs was dissolved in 1% w/v acetic acid at concentrations of 0.1, 0.2 and 0.3% w/v and kept under magnetic stirring until complete dissolution. Elevation of Cs solution pH to 6.2 was performed by the addition of appropriate volumes of 1 N NaOH, such pH value maintains Cs in its soluble form at all examined concentrations. TPP solution was prepared at different concentrations: 0.01, 0.02, 0.03, 0.04 and 0.05% (w/v). Both solutions were filtered through a 0.22 μm pore size filter (Millipore, Bil-lerica, USA). The Cs-TPP NPs were formed spontaneously by the dropwise addition of TPP solution onto Cs solution using 3 variable mass ratios (5:1, 7:1 and 9:1) of Cs and TPP, respectively. The gelation process was carried out under constant magnetic stirring (Yellow line MAG HS7, IKA, Germany) (500 rpm) at room temperature.

2.4. Characterization of Cs-TPP NPs

2.4.1. PS and ZP determination

Mean hydrodynamic PS, PDI and ZP measurements were performed on all Cs-TPP NP samples at a temperature of 25 ± 0.5 °C using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., UK). For PS analysis, a dynamic light scattering (DLS) technique was applied while Laser Doppler Anemometry (LDA) was used for ZP measurements. All samples were analyzed in suspension immediately after preparation in order to avoid changes in PS and charge due to Ostwald ripening or particle growth [15]. The mean of three measurements was calculated for PS, PDI or ZP.

2.4.2. Determination of Cs-TPP NPs yield

Being the best separation condition investigated giving the highest amount of particle recovery, the centrifugation technique was applied [16] using a cooling micro-centrifuge (Hermle Labortechnik GmbH, Model Z216 MK, Germany) adjusted at a speed of 15000 rpm under cooling conditions at 4 °C for 2 h. It is worthy to note that such centrifugal conditions applied for NP separation had no influence on the solubility of the individual Cs solution adjusted to pH 6.2, within the inspected concentration range. This was recognized by the absence of Cs precipitation and pellet formation. The pellets, however, formed from Cs-TPP NP formulations were lyophilized then weighed. A 50 ml volume of Cs-TPP NP formulation was most suitable for the evaluation of yield by this gravimetric method and it was calculated as follows:

\[
\% \text{Yield} = \left( \frac{W_n}{W_w} \right) \times 100
\]

where W_n is the total weight of Cs-TPP NPs recovered and W_w is the total weight of Cs and TPP used in the formulation observation.

2.4.3. Morphological characterization

Morphological examination of the optimized Cs-TPP NPs was performed by High resolution-transmission electron microscopy (HR-TEM) (JEOL JEM-2100, Japan) with an acceleration voltage at 200 kV. All samples for TEM analysis were prepared by allowing a single drop of NP suspension to dry at room temperature on a carbon-coated copper meshwork without being stained [17,18].

2.4.4. Fourier transform-infrared (FT-IR) spectroscopy

FT-IR spectrophotometer (Nicole 6700, thermo scientific, USA) was employed to record IR spectra (range 4000–400 cm⁻¹) of both
raw materials, Cs and TPP, in addition to lyophilized Cs-TPP NP formulation, embedded in KBr pellets.

2.4.5. Differential scanning calorimetry (DSC)

The thermal properties of (2–3 mg) samples of Cs, TPP and lyophilized Cs-TPP NP formulation sample were investigated using a differential scanning calorimeter calibrated with indium (DC-60, Shimadzu, Japan). Samples were sealed in aluminum pans with lids and heated from 25 to 400 °C at a rate of 10 °C/min. under nitrogen flow at a rate of 25 ml/min.

2.5. Experimental design

A total experimental set of forty five Cs-TPP NP formulations were prepared using this novel Cs solution pH; the three input variables viz. Cs concentration (%w/v), TPP concentration (%w/v) and Cs:TPP mass ratio were considered. The prepared NPs were characterized in terms of PS, PDI, ZP and% yield, however only Cs-TPP NP size and% yield were considered as outputs for ANN modeling. Table 1 demonstrates the composition of the forty five Cs-TPP NP formulations and their corresponding physicochemical characteristics and% yield.

2.6. ANN modelling

2.6.1. Software tool

The ANN analysis was developed using MATLAB R2015a software (version 8.5). A multilayer feed-forward-back propagation neural network was used to model the complex nonlinear relation between inputs and outputs and to present the outputs as three dimensional (3-D) graphs instead of statistical models because of its powerful nonlinear mapping capability.

2.6.2. Neural network architecture

The architecture of the ANN used in this study consists of three separate layers, an input layer, one hidden layer and an output layer. For modeling, a trial and error approach was performed to select the optimum number of neurons in the hidden layer, starting from the minimum number up to the smallest number of neurons generating a network with high predictability while sparing the occurrence of overfitting. The optimum number of neurons was selected at 7, in view of minimum root mean squared errors (RMSE), for both PS and% yield modeling. The topology of the ANN architecture used is shown in Fig. 1A.
2.6.3. Training, test and validation

Different training parameters were employed to obtain the best predictive network. Fig. 1B depicts that a Tanh transfer function (Tanh) was employed at the hidden and output layers and that the Levenberg–Marquardt learning method was used as the learning function for the formed networks, whilst the incremental back propagation (IBP) algorithm was applied for network training.

Two approaches were adopted to prevent over training of the network. The maximum number of iterations was set to 1000 (i.e. default value), in addition to splitting of experimental data into training, test and validation subsets. The test data set, consisting of 10% of the total experimental data set as recommended by the software, is taken, randomly, out of the training process to evade over training. The training process of ANN model stopped when the correlation coefficient of the test data starts to decrease, indicating that the network is becoming overtrained [6].

The “training” data set, composed of 30 experimental data points, was used to establish the cause-effect relationships, while the five data points, defined as “test” data, were used to evaluate the quality of the model and prevent overtraining. In addition, a set of ten experiments were excluded from training as “unseen” or “validation” data to assess the predictability of the generated ANN model. The quality of training and the predictive capability of the ANN models were evaluated by using RSME and correlation coefficients ($R^2$) and for training, test and validation data; with better predictability indicated by ($R^2$) values closer and closer to 1 [19]. The RSME and ($R^2$) were calculated using Eqs. (1) and (2), respectively [20].

\[
RSME = \left( \frac{1}{n} \sum_{i=1}^{n} \left( y_i - y_p \right)^2 \right)^{1/2} \tag{1}
\]

\[
R^2 = 1 - \frac{\sum_{i=1}^{n} \left( y_i - y_p \right)^2}{\sum_{i=1}^{n} \left( y_i - \bar{y} \right)^2} \tag{2}
\]

where $y_i$ is the mean of dependent variables (experimentally observed) and $y_p$ is the predicted value from the model. The selected network is the one with the highest $R^2$ and lowest MSE for all training, test, and validation data. The difference between the actual value of output and the predicted value for each data set is recorded as an error. Table 2 demonstrates the experimental, predicted and error values of the two examined outputs; PS and\% yield, in the training, test and validation data sets.

From the selected networks, it was possible to determine the relative effect of the input variables on the value of output. This was done by calculating the “contribution” of ith input data to kth output data ($C_{ik}$) for every input variable according to the following Eq. (3) [21]:

\[
C_{ik} = |\sum a_{ik} \times b_{jk}|
\]  

3. Results and discussion

Cs NP preparation by ionotropic gelation is highly sensitive to numerous environmental variables, as well as component and process conditions. The preparation process of Cs-TPP NPs involves optimization of polymer molecular weight, degree of decetylation, concentration, pH and ionic strength of its solution as well as TPP solution pH and concentration, Cs-TPP mass ratio, stirring rate and temperature conditions; to obtain positively charged Cs-TPP population of tunable charge and size [22,23].

In this research work, low molecular weight chitosan with highest degree of decetylation was selected to favor electropositive smaller sized Cs-TPP NPs preparation; as demonstrated in previous literature [24–26]. The experimental temperature and stirring rate were kept constant. In the meantime, based on previous work presented by our team [5], it was found that an elevation in pH values from 3 to 5 of both Cs and TPP solutions is accompanied by a general decrease in the PS, PDI and surface charge (ZP) of the NPs formed, at all examined mass ratios (from 1:3 to 5:1 w/w). While the NP yield of such formulations was very low, as NPs were difficult to be separated by centrifugation and hence to be used in gravimetric measurement of their yield. On the contrary, when polyamionic TPP was utilized at its initial pH 9.8, it was observed that this condition favored Cs-TPP NP diminution and yield% improvement, especially when Cs pH raised from 3 to 4 and 5. It was explained that TPP pH 9.8 allowed for the complete ionization of its anionic phosphate moieties ($pK_{a1} = 1$, $pK_{a2} = 2$, $pK_{a3} = 2.79$, $pK_{a4} = 6.47$, $pK_{a5} = 9.24$) enhancing its cross-linking efficiency and consecutively improving NP yield and PS diminution. The highest examined Cs and TPP solution pH values 5 and 9.8 respectively, showed increasing level of opalescence and yield% corresponding to 17.1, 22.3, and 29.4%, at mass ratios 9:1, 7:1 and 5:1 (w/w), respectively.

Therefore, in the present study, Cs and TPP solution pH elevation to values fixed at 6.2 and 9.8, respectively, was adopted for surplus boosting of process yield. The novel pH value 6.2 was expected to enhance the cross-linking of polymer chains into more NP formation as a result of the reduced electrostatic repulsive forces and amplified inter/intra chain H-bond linking at this pH value [27]. Nevertheless, Cs and TPP concentrations and mass ratios were examined in this research work for their effect on prepared NP characteristics and yield. A preliminary study was carried out to evaluate the levels of the three tested variables with regard to production of electropositive Cs-TPP NPs at maximum yield and
avoiding particle aggregation. Considering Cs solution concentration, it was observed that 0.05% w/v was not potential for NP formation while 0.4% was not soluble at investigated Cs solution pH. However, Cs solubility test confirmed complete Cs solubility (99.67 ± 2.11%) at our investigated pH 6.2 for 0.3% w/v solution. As for TPP concentration; 0.01–0.05% concentration range was selected for experimentation above which complete particle aggregation occurred. In the meantime, Cs:TPP mass ratios 5:1:7:1 and 9:1 were demonstrated in our previous research work to improve NP yield and size diminution in comparison to higher mass ratios; 13:1 and 11:1 [5].

3.1. Cs-TPP NP characterization

Results displayed in Table 1 demonstrate that the entire Cs-TPP NPs formulations prepared at pH 6.2 have mean hydrodynamic PS ranging from 162.7 ± 84.75 nm and PDI values in the range of 0.206–0.511. The stability of all prepared formulations was manifested by the absence of aggregates. In the interim, the entire Cs-TPP NP formulations carried positive surface charge ranging from +14.43 to +25.46 mV, indicating the presence of freely ionized amino groups on the surface of the nano-delivery systems which is important to allow for electrostatic repulsion between particles producing stable nano-dispersions. It is worthy to note the significant increase in mean particle surface charge (p < 0.05, Student's t-test) from +17.13 ± 1.44 mV to +21.26 ± 2.16 mV when Cs concentration is raised from 0.1 to 0.3% w/v, respectively. The final surface charge of Cs-TPP NPs prepared is impacted considerably by Cs concentration, which defines the amount of available- NH3+ moieties on the surface of NPs after NP formation.

Morphological characterization by HR-TEM for Cs-TPP NPs show compact nanoarchitectures of spherical morphology, as obvious in Fig. 2A, with relatively diminished size when compared to the measured hydrodynamic PS by DLS technique, ascribed to the dehydrating effect of TEM imaging as previously reported [4].

The intermolecular interaction of NPs was investigated by FTIR spectroscopy (Fig. 2B). Six characterization peaks, observed in Cs at 3321, 1655, 1587, 1423, 1371, and 1068 cm⁻¹, were thought
to reflect O–H stretching, C=O stretching from amide I, N–H bending and C–N stretching from amide II, –CH2 bending, –CH3 symmetrical deformation, and skeletal vibration of C–O stretching, respectively. This is in accordance with previous observations of Lawrie [28]. Concerning TPP IR spectrum, the peak at nearly 900 cm⁻¹ corresponds to P=O and P–OH stretching.

It was observed that the spectrum of Cs-TPP NPs was different from that of Cs matrix (Fig. 2B), highlighted in the wave number range from 1500 to 3500 cm⁻¹. The peak at 3321 cm⁻¹ became wider and flatter [18], as well those of amide I and amide II in Cs-TPP NPs were shifted to 1647 and 1531 cm⁻¹, respectively. Furthermore, the appearance of a peak at 885 cm⁻¹ that could be related to TPP, confirming the presence of the TPP moiety in the IR spectrum of Cs-TPP NPs.

Applying DSC investigation; it was found that TPP is an amorphous cross-linker showing no specific thermal transitions, as illustrated in Fig. 2C. On the contrary pure Cs DSC thermogram showed an endothermic peak at 68 °C, often termed as dehydration temperature (TD), due to the evaporation of water associated with the amorphous Cs chain segments and responsible for the strength of water-polymer interaction [29–32]. Additionally, an exothermic peak appeared at 310 °C possibly linked to polymer thermal decomposition [33,34]. The DSC scan of polymeric NPs demonstrated the two specific prominent peaks, yet shifting in the endotherm to nearly 90 °C and a broadening of the exothermic transition in range of 222–320 °C; with a specific lowering in thermal decomposition peak to 251 °C. The variation in TD of Cs-TPP NPs is believed to be due to the physical and molecular changes caused by the molecular interaction between Cs and TPP, suggesting the formation of more packed complexes [29]. Furthermore, the interactions between Cs and TPP restrained and hindered the movement of Cs molecules and destroyed orderliness of molecular chains, so that Cs crystallinity was suppressed [35]. This, in turn, was accompanied by a change in Cs thermal behavior manifested by an alteration in shape and position of the polymer degradation peak thus confirming the electrostatic complexion between Cs and TPP in the formed NPs.

### 3.2. Determination of the factors controlling Cs-TPP NP size using ANN

A neural network composed of three-layered feed-forward back propagation (3:7:1) was used to model the effect of input variables (Cs concentration, TPP concentration and mass ratio of Cs and TPP) on PS of the formed NPs. The best predictive model gave 𝑅² and RMSE values of 0.9835 and 14.54, 0.9658 and 6.55, and 0.952 and 19.13 for training, test and validating data, respectively. The overall 𝑅² for the model was 0.974, as shown in Fig. 3, indicating the quality of the trained network and its high predictability. Table 2 tabulates the measured PS by DLS technique, the predicted PS and error values calculated by the ANN.

<table>
<thead>
<tr>
<th>Experimental parameter</th>
<th>Cₐ for PS</th>
<th>Cₐ for % yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPP concentration</td>
<td>9.767</td>
<td>16.206</td>
</tr>
<tr>
<td>Cs concentration</td>
<td>8.54</td>
<td>4.801</td>
</tr>
<tr>
<td>Cs:TPP mass ratio</td>
<td>8.142</td>
<td>5.9964</td>
</tr>
</tbody>
</table>

The relative contribution of each input variable in PS, expressed by Cᵦ, was determined (Table 3). It was found that the TPP concentration was the most influential factor, as it has the highest Cᵦ value, followed by Cs concentration, and then Cs:TPP mass ratio. The ANN created a nonlinear 3-D mapping for the effect of the input variables on NP (Fig. 4).

These 3-D surfaces showed the strong effect of TPP concentration; which was directly correlated with PS enlargement. It can be explained that the use of diluted solutions of TPP promotes the dilution of the formulation medium, promoting Cs-TPP interaction into the formation of a more homogenous population of compact particles [18]. Moreover, this dilution generates spatial distance between polymer chains and between neighboring particles formed thereby decreasing the incidence of cluster formation (particle growth).

The effect of Cs:TPP mass ratio on PS was shown in these 3-D models to vary according to Cs and TPP concentrations used. The 9:1 and 7:1 mass ratios were most optimum for the preparation of small sized CsNPs at 0.3% and (0.1 and 0.2%) w/v Cs solutions, respectively using TPP concentrations (0.02–0.05% w/v). With an exception of
0.01% w/v TPP concentration; Cs: TPP mass ratio didn’t influence NP size at 0.1 and 0.2% Cs concentrations but a 5:1 mass ratio caused most noticeable PS reduction when 0.3% Cs concentration was used.

3.3. Determination of the factors controlling Cs-TPP NP% yield using ANN

Another neural network similarly composed of three-layered feed-forward back propagation (3:7:1) was used to model the effect of input variables (Cs concentration, TPP concentration and mass ratio of Cs and TPP) on the% yield of the formed NPs. Thirty individual data sets were randomly selected as “training data”, while five individual data sets were randomly selected as “test data” and ten other individual data sets were randomly selected as “validating data”. This model showed $R^2$ and RMSE values of 0.9997 and 3.651, 0.969 and 1.693, and 0.9987 and 6.501 for training, test and validating data, respectively. The overall $R^2$ for the model was 0.9965, indicating its high predictability (Fig. 6).

$C_{ik}$ values were computed and it was found that the effect of factors on% yield was in the order of TPP concentration > Cs:TPP mass ratio > Cs concentration, as shown in Table 3. The $C_{ik}$ results were supported by the 3-D surface plots of the predicted% yield from the ANN model (Fig. 5). These plots illustrated an increase in yield accompanying the decrease in TPP concentration up to 0.02% w/v, after which a significant reduction in process yield is obtained, this behavior is observed whatever Cs concentration was employed. It is clear from afore-mentioned explanations that the diluted TPP solution (0.01 and 0.02% w/v) decreases the viscosity of the gelation medium potentiating Cs-TPP interaction [18] which was found to enhance NP productivity and process yield. However, the TPP

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Fig. 3. Actual versus predicted particle size from network and regression for training, test, validation and all data sets.

Fig. 4. 3-D surface plot of particle size predicted by ANN model at different TPP concentrations and Cs: TPP mass ratios while keeping Cs concentration constant at (A) 0.1% w/v, (B) 0.2% w/v and (C) 0.3% w/v.
4. Conclusion

In this study, we succeeded to prepare Cs-TPP NP at a novel Cs solution pH 6.2 and maximize yield to over 90% by optimizing Cs and TPP concentrations and mass ratios. The ANNs approach was capable of generating a quality model representing the formulation of Cs-TPP NPs at this uninvestigated Cs solution pH. The developed model was validated statistically and exhibited high predictive capability. TPP concentration was found to be the more dominant factor in controlling Cs-TPP NP size and process yield. Data generated from the ANN model in the form of 3D graphs were used to indicate the important interactions between input variables and the output. Eventually, the multivariate trained models create deeper understanding and knowledge of parameters affecting the PS and process yield of Cs-TPP NPs. Such models, in addition, can aid in prediction and optimization of processing conditions and outcomes.