Green approach for fabrication of chitosan-neem gum polyelectrolyte stabilized penta and hexagonal nanoparticles and in-vitro cytotoxic potential toward breast cancer (MCF-7) cells

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Abstract

The present investigation aimed to utilize chitosan-neem gum polysaccharide (Ch-NGP) polyelectrolyte complex for the fabrication of hexagonal and pentagonal nanoparticles using antisolvent precipitation method. Fabricated nanoparticles were found in the range of 63.1 to 447.2 nm with the entrapment efficiency of 76.80 ± 1.28 to 89.82 ± 2.32%. A nonlinear correlation between the independent variable and response was observed after the regression coefficient based linearity analysis. Drug release was carried out using the egg membrane and tomato membrane as a biological barrier. All the formulations show peculiar release pattern viz. initial immediate release, followed by sustained release and final burst release of the drug. Similarity factor analysis easily showed a significant difference in drug release patterns when the egg membrane and tomato membrane were utilized as biological barriers (S<50). Anticancerous effect against breast cancer cells line (MCF-7) shows better control over cell growth when etoricoxib loaded nanoparticles were used in place of pure etoricoxib. Particle size growth analysis elicits that significantly no “Ostwald ripening” was observed after 45 days. It can be concluded from the findings of the experiments that Ch-NGP polyelectrolyte functionalized hexagonal and pentagonal nanoparticles can be utilized for passive targeting of tumor cells due to its unique properties.

KEYWORDS

antisolvent precipitation, chitosan, cytotoxicity, etoricoxib, nanoparticles, neem gum polysaccharide, polyelectrolyte complex

1 | INTRODUCTION

Solubility is a profound decision-making parameter for the development of pharmaceutical formulation. Understanding of dose-related toxicity is important for pharmaceutical scientists. In 2012, Morgan et al have identified that poor aqueous solubility is a major cause of drug failure during clinical trials.¹ Poor aqueous solubility is a key obstacle to the therapeutic effect of API. Solubility hence dissolution of API is a driving force for drug absorption.² The low aqueous solubility of drug hampers their bioavailability when orally administered. A new synthetic chemical entity may fail during the product development process due to poor aqueous solubility.³,⁴ Among poorly watersoluble drugs, only 20% to 30% can undergo salt formation, so the
remaining 70% to 80% must go through another route of solubility enhancement for improved therapeutic effect. Conventional solubility enhancement methods such as emulsion, micelles formation, and cyclodextrin complex are not always effective and required a relatively large quantity of excipients.

Numerous experimental, epidemiologic, and clinical studies suggest that nonsteroidal anti-inflammatory drugs (NSAIDs), particularly the highly selective cyclooxygenase COX-2 inhibitors, have promise as anticancer agents. NSAIDs have been shown experimentally to stimulate apoptosis and to inhibit angiogenesis, two mechanisms that help to suppress malignant transformation and tumor growth. The poor aqueous solubility of COX-2 inhibitors limits their potential to treat cancer. The improved aqueous solubility of hydrophobic drugs also facilitates oral bioavailability. Any methods that improve their aqueous solubility also enhance their therapeutic effect.

Significant advancement in pharmaceutical engineering opens a new area of nanoparticle preparation known as “nanoprecipitation.” The author’s had discussed nanoprecipitation in the previous research. In continuation of previous work, here researcher used chitosan and NGP based polyelectrolyte complex (PEC) as a stabilizer for the nanoprecipititates of poorly water-soluble drug etoricoxib. This method includes the simultaneous interfacial deposition of PEC with the rapid migration of the solvent. It maximizes drug loading and improves entrapment efficiency.

The interaction between oppositely charged ions in aqueous solution leads to the formation of PEC. The coulombic interaction between ions leads to the formation of a complex structure having unique physical and chemical properties. The transition of ionic groups to form PEC develops a new platform to induce desirable properties into formulations. PECs are biocompatible, biodegradable and have superior properties than native polymers.

Chitosan (Ch), a natural polysaccharide is made up of β-(1-4) linked β-glucosamine (deacetylated unit) and N-acetyl-β-glucosamine (acetylated unit). It is a commonly used cationic polymer for the preparation of PEC. Various polysaccharides such as guar gum, gum acacia, locust bean gum, pectin, and so on have been used as an anionic polymer for the PEC formation. Below pH 6.5, the amino groups of Ch become ionized and net positive charge appears over chitosan structure. It induces capabilities to bind –ve charge of polysaccharide in aqueous solution. Physicochemical properties of PEC depend upon the charge density; the position of chargeable groups, polymeric chain length, chain flexibility, and so on. Monitoring of pH for the ionization of polymers becomes essential especially in the case of low charge density-based polymers. Change in concentration of ionic polymer leads to the formation of PEC having different crosslinking density. Crosslinking density between oppositely charged polymers determines the stability, pH sensitivity, solubility, physical strength, porosity, swelling characteristics, and drug release pattern. In present research polymeric PEC has been utilized to improve the dissolution of BCS Class-II drug etoricoxib.

Neem gum polysaccharide (NGP) is a branched-chain gum exudate from the tree Azadirachta indica, family Meliaceae. Hydrolysis of gum shows the presence of l-arabinose, l-fucose, d-galactose, d-glucuronic acid, and traces of d-xylene. The ratio of d-galactose to l-arabinose was found to be 3:2. They are present as furanose forms within the gum. NGP has been used as antioxidant, emulsifying agent, binder, and film coating material.

In present work, Ch and NGP were used as cationic and anionic polymer, respectively for the preparation of PEC. Formation of PEC due to the interaction of –NH₃⁺ group of chitosan and –O⁻ group of NGP may be a promising emerging combinational polymeric system for biomedical as well as pharmaceutical applications. The initiation of interaction between oppositely charged ions does not require any specific catalyst or crosslinker. These characteristics eliminate the use of additives and reduce associated toxicity along with the total cost.

As per our best knowledge, it was the first attempt to study the effect of the ratio of Ch and NGP oversize and entrapment efficiency of nanoparticles. Hexagonal and pentagonal nanoparticles have been prepared but not explored as a drug delivery carrier. Highly faced (hexagonal and pentagonal) nanoparticles were prepared and used as drug delivery carriers for cancer therapy.

## 2 | MATERIALS

Chitosan (molecular weight: 190000-310 000 Da) was procured from Merck Specialties Private Limited, Mumbai India. Ethyl alcohol and acetone were supplied by SD. Fine Chemicals Mumbai India. All the chemicals were used as supplied, without any purification. In experiments, HPLC grade water was used. Drug etoricoxib was obtained as a gift sample from Cipla Ltd Mumbai.

As described in our previous publication, crude NGP was collected and purified using a water-based extraction process.

## 3 | METHODS

### 3.1 | Factorial design

PEC stabilized nanoparticles were prepared using 3² factorial design. In the present research concentration of Ch and NGP were selected as independent variables while particle size and entrapment efficiency were as dependent variables (response factor). Three levels were selected for each independent variables as shown in Table 1 and the result was analyzed using NCSS 12 software (Trail version May 15, 2018).

### 3.2 | Preparation of PEC stabilized nanoparticles

In the present study, nanoparticles were fabricated using a non-stoichiometric ratio of cationic and anionic polymers as shown in Table 1. Ch and NGP solutions (20 mL) were prepared separately by using 1% acetic acid and water as the solvent, respectively. NGP
solution was transferred dropwise into Ch solution and stirred at 50 rpm and 45°C for 30 minutes (PEC solution). Drug solution (10 μg/mL) was prepared using acetone as the solvent and added dropwise into PEC solution using a syringe (BD Emerald 5 mL). Furthermore, the solution was stirred for 30 minutes at 45°C and sonicated for 10 seconds. After that, the sample was cooled to 35°C and stored in an airtight glass container for further study.

### 3.3 | FTIR spectral analysis

FTIR (Alpha, ECD-ATR) analysis was performed to investigate any possible interaction between polysaccharides and polysaccharide-based PEC and drug.

### 3.4 | DSC analysis

DSC (DSC-60, Shimadzu, Japan) of etoricoxib and nanoparticles (N5) were performed to analyze the solubility behavior of pure etoricoxib and optimized formulation N5. The analysis was performed under a nitrogen atmosphere at the heating rate of 10°C/min with nitrogen purging rate at 50 mL/min.

### 3.5 | Evaluation of PEC stabilized nanoparticles

Ch-NGP PEC based nanoparticles were characterized in terms of the following parameters:

| Formulation | Ch (%w/v) | NGP (%w/v) |
|-------------|-----------|------------|
| N1          | 0.03      | 0.03       |
| N2          | 0.03      | 0.01       |
| N3          | 0.03      | 0.02       |
| N4          | 0.01      | 0.03       |
| N5          | 0.01      | 0.01       |
| N6          | 0.02      | 0.02       |
| N7          | 0.02      | 0.01       |
| N8          | 0.02      | 0.02       |
| N9          | 0.02      | 0.02       |

### 3.6 | Particle size and surface potential analysis

PEC stabilized nano-suspensions were diluted to prepared 1%v/v suspension and size and zeta potential were determined using zeta seizer (Malvern instrument, Version 6.32, Model No. ZEN3500, United Kingdom).

### 3.7 | SEM analysis

Surface characteristics and shape of formulated nanosuspension were analyzed using Zeis EVO analyzer (Malvern instrument, Version 6.32, Model No. ZEN3500, United Kingdom).

### 3.8 | Loading efficiency (%)

Formulation was mixed with 10 mL of 0.1 N HCl for 2 hours. The whole solution was centrifuged at 16,000 rpm in REMI centrifuge (CM-8 plus, India) at 15°C for 30 minutes. The clear supernatant solution of drug content was analyzed using Shimadzu UV visible spectrophotometer (UV-1800, Shimadzu, Japan). The percentage of drug loading was calculated by using the following Equation (1):

\[
\text{Drug loading} (%) = \left( \frac{\text{Amount of drug found in the nanoparticles}}{\text{Amount of nanoparticles}} \right) \times 100
\]

### 3.9 | In vitro drug release

In vitro drug release of formulations was determined by using the rotator basket (USP Type I) dissolution apparatus (Lab India Dissolution Tester: DS 8000+, India). Nanosuspensions were enclosed in the tomato membrane and egg membrane (acts as biological membrane). Drug release was carried out in 0.1 N HCl for 120 minutes followed by a study in phosphate buffer pH 7.4. The temperature of the medium was kept at 37 ± 0.5°C and basket rotation was maintained at 50 rpm. Aliquots were withdrawn, filtered using Whatman filter paper (pore size 20 nm) and % drug release was calculated. Results were shown as an average of triplicate studies with SD.

### 3.10 | Preparation of egg membrane

Egg of chicken (Gallus gallus) was taken to prepare the egg membrane. An orifice was made at one end of the egg and through an opening, the yolk was completely removed. The shell of the egg was kept in a beaker containing acidified water. The temperature was raised to 45 ± 2°C and bubble formation takes place. After some time bubble formation stop and the foam gets vanished. After that only membrane was left in the beaker as the eggshell contains calcium carbonate that releases as foam when it comes in the contact of acidified water (2).

\[
\text{CaCO}_3 + 2 \text{HCl} \rightarrow \text{CaCl}_2 + \text{CO}_2 \uparrow + \text{H}_2\text{O}
\]

### 3.11 | Preparation of tomato membrane

A cross was made at the top of the tomato (Solanum lycopersicum) to release the pulp and kept in a beaker containing water.
temperature of the beaker was raised to 45 ± 2°C and after 20 minutes; the membrane was slowly and carefully removed, washed and utilized for further study.

### 3.12  |  Kinetics of drug release

It is always necessary to determine the release characteristics of the drug from the formulation. In the present research model dependant methods viz. Zero-order, First order, Higuchi model, Korsmeyer-Pepass model, Hixon-Crowel model, and Baker-Lonsdale model and model-independent methods viz. the similarity factor was applied to characterize drug release pattern. In the present study, optimized formulations were selected and a model-dependent method was applied to characterize drug. Zero-order drug release can be shown by Equation (3).

\[
Q' = Q_o + K_o t
\]  

(3)

where \( Q' \) is the amount of drug dissolved at time \( t \). \( Q_o \) is the initial amount of drug in solution and \( K_o \) is zero-order release constant.

First-order release kinetics is expressed by Equation (4), where \( C_o \) is the initial concentration of drug in solution and \( C \) is the concentration of drug at time \( t \) and \( K \) is first-order rate constant.

\[
\log C = \log C_o - \frac{kt}{2.303}
\]  

(4)

Higuchi kinetic model is shown by Equation (5)

\[
Q = K_{H} t^\frac{1}{2}
\]  

(5)

Where \( K_{H} \) is Higuchi dissolution constant, \( Q \) is the amount of drug diffusion to the solution at time \( t \). Equation (6) is used to show the Korsmeyer-Pepass model of drug release.

\[
\frac{M_t}{M_w} = K t^n
\]  

(6)

Where \( \frac{M_t}{M_w} \) is the fraction of drug released at time \( t \), \( K \) is release rate constant and \( n \) is release exponent.

Mathematically, Hixon-Crowel release kinetics can be expressed as Equation (7).

\[
W_3^t - W_3^0 = k t
\]  

(7)

Where, \( W_o \) is the initial amount of drug in pharmaceutical dosage form, \( W_t \) is the remaining amount of drug in the pharmaceutical dosage form at time \( t \) and \( k \) is a constant incorporating the surface volume ratio.

Baker-Lonsdale’s model of drug release can be expressed as Equation (8).

\[
f_1 = \frac{3}{2} \left[ 1 - \left(1 - \frac{M_t}{M_w} \right)^3 \right]^\frac{1}{3} = k t
\]  

(8)

Where, the release rate constant, \( k \), corresponds to the slope.

### 3.13  |  Model-independent approach using similarity factor

Scale-Up and Post Approval Changes (SUPAC) guidelines provide a mathematical tool that is, similarity factor \( S \), for the comparison of dissolution profile of two formulations. Similarity factor \( S \) measures the closeness between the dissolution profiles of formulations. FDA and EMEA defined similarity factor as "logarithmic reciprocal square transformation of one plus the mean squared difference of drug percent dissolved between the test and the reference products."

In present research dissolution profiles of formulation N5 using egg membrane and tomato membrane were characterized in terms of difference factor \( (f_1) \) and similarity factor \( (S) \). Difference factor \( (f_1) \) defines the percent difference in drug release between two curves at the same time (Equation (9)).

\[
f_1 = \left( \frac{\sum_{n=1}^{N} |R_t - T_t|}{\sum_{n=1}^{N} R_t} \right) \times 100
\]  

(9)

Where \( n \) is the number of time points, \( R_t \) is dissolution value of N5 when egg membrane was used as a biological barrier as time \( t \) and \( T_t \) is dissolution value of N5 when tomato membrane was used as a biological barrier at same time \( t \). The similarity factor is log reciprocal square root transmission of the sum of square error (Equation (10)).

\[
\text{Similarity factor}(S) = 50 \log \frac{1}{\sqrt{f_1 + f_2 + f_3 + \ldots + f_n}}
\]  

(10)

During the literature survey, it was found that the Similarity value \( S \) is a well-established parameter for the comparison of drug release patterns between two formulations. If the value of \( S \) is 100, it means both the formulations are the same. \( S \) value between 50 and 100 indicates a similarity between two dissolution profiles. The value under 50 indicates significant differences between the release profiles of two formulations. The \( f_1 \) value should be close to 0 and \( S \) should be close to 100 for similar formulations. Similarity factor analysis was used to identify either release pattern of etoricoxib was the same or not when two different biological membranes viz. tomato membrane and egg membrane, were used as biological membranes.

### 3.14  |  Particle size analysis

Formulated nanoparticles are in direct contact with the solvent system, that is, aqueous medium. So, it becomes necessary to evaluate the effect of the presence of the solvent in crystal growth. Crystal
growth is an undesirable phenomenon for any nanoformulation. Formulations were withdrawn at regular intervals viz. 7, 14, 30, and 45 days and the effect of “Ostwald ripening” or crystal growth was analyzed using zeta sizer.

3.15 | Cytotoxicity screening

In vitro cytotoxicity study of pure drug and optimized formulation N5 was carried out against breast cancer cell lines (MCF-7) at anti-cancer drug screening facility (ACDSF) at ACTREC, Tata Memorial Centre, Navi Mumbai. For the activity, SRB (sulphorhodamine B) was employed.

In the present study, MCF-7 cells were incubated at 37°C into 96 well microtiter plates for 24 hours. After the inoculation of the cell, before the addition of experimental drugs the microtiter plates were incubated with 5% CO₂, 95% air, and 100% relative humidity for 24 hours at 37°C. Test samples were initially solubilized in dimethyl sulfoxide at 100 mg/mL and diluted to 1 mg/mL using water and stored frozen before use. An aliquot of frozen concentrate (1 mg/mL) was thawed and dilutions of 2, 4, 8, and 10 μg/mL solution of test samples were prepared. After the addition of the sample, the plates were incubated for 48 hours at standard conditions and reaction was terminated by the addition of cold trichloroacetic acid (TCA). By the gentle addition of 50 μL of cold 30% (w/v) TCA (final concentration, 10% TCA) the cells were fixed and further incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were five times washed with tap water and air-dried. At room temperature Sulforhodamine B (SRB) solution (50 μL) at 0.4% (w/v) in 1% acetic acid solution was done and the plates were dried. With 10 mM trizma base the bound stains were subsequently eluted and at a wavelength of 540 with 690 nm reference wavelength, the absorbance was read on a plate reader.

4 | RESULTS

No interaction was observed after FTIR spectral analysis of drug etoricoxib and NGP-Ch PEC (Supporting Information). IR spectra of etoricoxib show characteristic peaks at 1590 cm⁻¹ (C=N stretch), 3350 cm⁻¹ (O-H) and 1299 cm⁻¹ (S=O). IR spectra of Chitosan shows characteristics peaks at 1590 cm⁻¹ (C=N stretch), 639 to 670 cm⁻¹ (Cl-C stretch), 3616 cm⁻¹ (O-H Stretch), 2361 cm⁻¹ (COOH), and 2115 cm⁻¹ (C≡C stretch). In the IR spectra of NGP, a peak appears at 1728 cm⁻¹ due to stretching vibrations of –C=O. The peak near 2960 appears due to –C–H stretching of –C–O–CH₃. The peak at 1325.43 cm⁻¹ wave number appears due to asymmetric stretching of the cyclic –C–O–C region in the molecule. It was found during the study that –C–H deformation in –CH₂ shows a peak between 1440 and the same was confirmed due to 1442.81 cm⁻¹ peak. Characteristic wave numbers present in spectra were 1728.98 (stretching vibrations of –C=O), 2960 (–C–H stretching of –C–O–CH₃ (methyl ether), 1325.43 (asymmetric stretching of cyclic –C–O–C), 1442.81 and 1350.72 (–C–H deformation in –C≡C), IR spectra of nanoparticles N5 showed characteristic peaks of 1728 cm⁻¹ (C=O Stretching), 1325.43 cm⁻¹ (C=O=C), 1442.81 cm⁻¹ (CH₃), 3616 cm⁻¹ (O–H Stretching), 2361 cm⁻¹ (COOH), 1299 cm⁻¹ (S=O), 1515 cm⁻¹ (N–H Bend), 1590 cm⁻¹ (C≡N Stretching), and 639-670 cm⁻¹ (C–Cl Stretching). No extra peak was observed in the

![FIGURE 1](image1.png) **FIGURE 1** Surface plot for effect of independent variables over size of nanoparticles

![FIGURE 2](image2.png) **FIGURE 2** Surface plot for effect of independent variables entrapment efficiency of nanoparticles
spectra of N5, so it can be concluded that interaction was not taken place between the etoricoxib and NGP-Ch PEC.

4.1 | DSC analysis

DSC thermogram of etoricoxib and formulation N5 can be found as Supporting Information. As discussed by authors in the previous study, DSC thermogram of NGP showed endothermic peaks at 57.50°C, which originated due to pyrolysis of NGP initiated by the random breakdown of glycosidic bonds and shows its amorphous nature.8 DSC thermogram of etoricoxib shows the melting point at 132.95°C. This peak of etoricoxib confirmed its crystalline nature as discussed by Das et al.22 DSC thermogram of Formulation N5 showed two peaks at 59.2 and 79°C. A peak at 79°C may be due to change in the crystalline form of etoricoxib into amorphous form while the peak at 59.2°C may be due to pyrolysis of polysaccharide. An amorphous form of the drug has a higher solubility than the crystalline form of the drug.

Polymeric PEC was water-soluble, biodegradable and use water as a solvent for the preparation. In the present investigation, polymers were required to be charged to facilitate PEC formation, so distilled water and 10% acetic acid solution was used as a solvent for the preparation of NGP and Ch solution, respectively. It also minimizes the side effects associated with organic solvents. Generally, nonaqueous solvents are used to improve the solubility of poorly water-soluble drugs, viz. micro and nanoemulsions. In this study oil, free polyelectrolyte nanosystems were used to encapsulate poorly water-soluble BCS class-II drug etoricoxib. Formations of PEC facilitate drug dissolution and promote paracellular drug transport. The hydrophobic drug was dispersed in the aqueous medium and encapsulated within the PEC structure.

Nanosizing of drug molecules increases Gibb's free energy. Drug nanoprecipitates start to form agglomerate to reduce their Gibbs energy. To prevent crystal growth, a stabilizer is required, which form a barrier over nanoprecipitates and prevent crystal growth and

| Table 2 | Characterization parameters of PEC stabilized nanoparticles |
|----------|----------------------------------------------------------|
| Formulation | Physical appearance | Particle size (nm) | Zeta Seizer | SEM | PDI | Zeta potential (mV) | Entrapment efficiency (%) |
| N1 | Clear solution | 447.2 | 358-388 | 0.362 | 32.2 | 83.38 ± 1.81 |
| N2 | Clear solution | 300.3 | — | 0.385 | 33.1 | 85.63 ± 1.57 |
| N3 | Clear solution | 377.1 | — | 0.365 | 32.6 | 76.80 ± 1.28 |
| N4 | Clear solution | 189.0 | 137-190 | 0.346 | 33.5 | 89.82 ± 2.32 |
| N5 | Clear solution | 63.1 | 46-51 | 0.354 | 33.4 | 78.56 ± 2.01 |
| N6 | Clear solution | 193.1 | — | 0.362 | 34.3 | 78.72 ± 1.61 |
| N7 | Clear solution | 186.0 | — | 0.298 | 35.2 | 78.67 ± 1.93 |
| N8 | Clear solution | 131.2 | 115-250 | 0.359 | 34.9 | 83.56 ± 2.34 |
| N9 | Clear solution | 88.0 | — | 0.312 | 33.6 | 83.52 ± 1.89 |

Note: Figure 3A was reproduced from reference 8 after permission
agglomeration. In the present experiment, Ch-NGP was used to form a PEC that was further utilized as a stabilizer.

4.2 Factorial design

In the present investigation, 3² full factorial design was employed to evaluate the effect of two independent variables that is, concentration of cationic polymer (Ch) and concentration of anionic polymer (NGP) on dependent variable that is, particle size and entrapment efficiency. The reduced equation to measure the response (particle size and entrapment efficiency) having statistical significance for 3² factorial design can be shown as below Equation (11).

\[ Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2 \]  

Where, \( Y \) is the response (dependent variable), \( b_0 \) arithmetic mean response of nine batches and \( b_1 \) estimated coefficient for factor \( X_1 \). The coefficients corresponding to linear effects (\( b_1 \) and \( b_2 \)), interaction (\( b_{12} \)) and the quadratic effects (\( b_{11} \)and \( b_{22} \)) were determined from the results of the experiment. \( X_1 \) and \( X_2 \) are the concentration of Ch and NGP, respectively.

![Graph](a) Drug release of formulations (N1-N5) using egg membrane as biological barrier

![Graph](b) Drug release of formulations (N6-N9) using egg membrane as biological barrier

**FIGURE 4** Drug release from nanoparticles using egg membrane as biological barrier. A, Drug release of formulations (N1-N5) using tomato membrane as biological barrier. B, Drug release of formulations (N6-N9) using tomato membrane as biological barrier
From the experimental data, Equation (11) was solved to generate the effect of concentration of Ch and NGP over particle size (Equation (12)) and entrapment efficiency (Equation (13)) of PEC stabilized nanoparticles.

\[
\text{Particle size (nm)} = 219.33 + 113.17(X_1) + 54.67(X_2) + 5.25(X_1X_2) + 28.11(X_{12}) + 0.00(X_{22}) \quad (12)
\]

As shown in Equation (12), quadratic constant \(X_{22}\) had no effect over particle size.

\[
\text{Entrapment efficiency (\%)} = 82.07 + 114(X_1) + 58.33(X_2) + 14.25(X_1X_2) + 0.05(X_{12}) + 0.80(X_{22}) \quad (13)
\]

The surface plot for the effect of independent variables oversize and entrapment efficiency of nanoparticles was depicted in Figures 1 and 2, respectively. A Scatter plot to show the effect of independent variables oversize and entrapment efficiency of nanoparticles was depicted in Supporting Information.

Linearity was analyzed using regression coefficient determination through straight-line Equation (14).

\[
Y = mX + C \quad (14)
\]

The graph was plotted between the independent variable and its response and the regression coefficient was determined to evaluate...
uniform and linear correlation between changes in response with independent variables (Supporting Information). A higher value of regression coefficient ($R^2$) for the effect of one independent variable than the response of another independent variable shows a better linear effect of that particular independent variable over response. As shown in Supporting Information, the concentration of chitosan ($R^2 = 0.567$) has a better linear effect over particle size than the concentration of NGP ($R^2 = 0.132$). No linear correlation was established between concentrations of chitosan ($R^2 = 0.002$) and concentration of NGP ($R^2 = 0.02$) over the entrapment efficiency of PEC stabilized nanoparticles.

Prepared PEC stabilized nanoparticles are capable to deliver drugs and have the potential to improve drug dissolution. Drug entrapment depends upon the ratio of polymers used and the polarizability of groups to form strong bonds. Various characterization parameters of prepared nanoparticles were shown in Table 2.

4.3 | Shape and size of nanoparticles

Shape and size of nanoparticles play a crucial role in their biomedical and pharmaceutical applications. Crystalline nanofluidic materials have a better dissolution rate due to the higher surface area to volume ratio. Particle size reduction below 1 μm improves salvation properties and increases solubility. Particle size reduction also results in disruption of solute-solute interaction that further leads to better dissolution.

In present investigation formulation N5 (concentration of Ch 0.01%w/v, concentration of NGP 0.01%w/v) shows lowest particle size that is, 63.1 nm while N1 shows largest particle size that is, 447.2 nm (concentration of Ch 0.03%w/v, concentration of NGP 0.03%w/v). It shows the direct effect of independent variables over the size of nanoparticles.

As shown in Figure 3A pure drug nanoparticles were crystalline and needle-shaped while PEC stabilized nanoparticles (Figure 3B) were hexagonal and pentagonal in shape.

4.4 | Dissolution and drug release

Drug release data shows the peculiar characteristics of drug release patterns of nanoparticles (Figures 4 and 5). Biological membrane (viz. egg membrane and tomato membrane) were not changed specific release patterns of drug. All the formulation shows three phases of drug release viz. initial immediate release followed by sustained release and final burst release. The time required to release 80% drug ($t_{80\%}$) was calculated for all the formulations and tabulated (Table 3). Minimum and maximum $t_{80\%}$ was found for N2 (94 minutes) and N1 (and N4) (129 minutes), respectively when the egg membrane was used as a biological membrane. Minimum and maximum $t_{80\%}$ was found for N3 (127 minutes) and N2 (and N7) (131 minutes), respectively when the tomato membrane was used as a biological membrane.

Egg membrane and tomato membrane both are used as biological barriers for drug release study to compare and identify alternate

### Table 4: Time dependent drug release study through the egg membrane

| Batch | Cumulative % drug release (in 45 minutes) | Cumulative % drug release (in 6 hours) | Actual % drug release (between 45 minutes and 6 hours) |
|-------|------------------------------------------|----------------------------------------|-------------------------------------------------|
| N2    | 39.32 ± 2.13                             | 99.89 ± 7.44                           | 60.57                                           |
| N3    | 64.91 ± 1.4                              | 99.97 ± 4.02                           | 35.06                                           |
| N4    | 57.36 ± 1.03                             | 98.99 ± 4.67                           | 42.63                                           |
| N5    | 39.97 ± 1.05                             | 98.97 ± 3.28                           | 59                                              |
| N6    | 60.22 ± 0.82                             | 99.98 ± 4.73                           | 39.76                                           |
| N7    | 60.91 ± 0.95                             | 99.88 ± 6.83                           | 38.97                                           |
| N8    | 54.81 ± 1.03                             | 99.96 ± 4.93                           | 45.51                                           |
| N9    | 50.76 ± 1.3                              | 98.89 ± 4.75                           | 48.13                                           |

### Table 5: Time dependent drug release study through tomato membrane

| Batch | Cumulative % drug release (in 45 minutes) | Cumulative % drug release (in 7 hours) | Actual % drug release (between 45 minutes and 7 hours) |
|-------|------------------------------------------|----------------------------------------|-------------------------------------------------|
| N1    | 62.38 ± 3.12                             | 99.98 ± 5.4                            | 37.6                                            |
| N2    | 78.36 ± 2.4                              | 98.99 ± 7.56                           | 20.63                                           |
| N3    | 62.32 ± 3.54                             | 99.99 ± 6.46                           | 37.67                                           |
| N4    | 71.30 ± 2.4                              | 98.98 ± 8.56                           | 27.68                                           |
| N5    | 73.47 ± 2.4                              | 99.88 ± 6.6                           | 26.41                                           |
| N6    | 70.00 ± 2.56                             | 98.97 ± 6.56                           | 28.97                                           |
| N7    | 58.33 ± 2.54                             | 99.96 ± 5.45                           | 41.63                                           |
| N8    | 71.19 ± 2.45                             | 99.98 ± 6.75                           | 28.79                                           |
| N9    | 66.25 ± 2.89                             | 98.98 ± 5.67                           | 32.73                                           |
Time of 80% of drug release ($T_{80}$) from the formulation was summarized as Table 3. Tables 4 and 5 summarized the time-dependent drug release data when egg membrane and tomato membrane, respectively, were used as biological barriers.

Kinetic model study becomes an essential parameter to determine the mechanism responsible for drug movement from formulation to the external medium. All the formulation follows Baker-Lonsdale kinetics due to higher regression coefficient value ($R^2$; Supporting Information).

The model-independent approach predicts the value of difference factor ($f_1$) and similarity factor (S) 23.45 and 34.25, respectively, when drug release was compared for formulation N5. In this approach, the egg membrane was used as a standard while the tomato membrane was a test biological membrane. Cumulative drug release at 45 minutes and 6 hours were selected as sampling point and corresponding values were considered for mathematical treatment. It can be concluded from the study that drug release pattern through two different biological membranes is dissimilar to each other.

### 4.5 Stability concern

Crystalline forms of nanoparticles are thermodynamically more stable than amorphous forms. Conversion of the amorphous form of the formulation into crystalline form during storage is one of the basic issues that must be considered before the formulation of nanosuspension as a drug delivery carrier. It can be concluded from the data provided in Table 6. That particle size was increases or decreases depending upon the formulation, while in all cases entrapment efficiency was decreased due to leaching of etoricoxib from the nanoparticles. Except for N6 and N8, all other formulation shows an increase in particle size due to crystal growth.

### 4.6 In-vitro cytotoxic study

Findings of the result showed that nanosuspension (N5) has better control over cancerous cells growth as compared to pure drug that is, etoricoxib (Figure 6). As the concentration increases, N5 showed more pronounced control over MCF-7 cells. Results easily predict that PEC stabilized etoricoxib nanoparticles can be used in place of pure etoricoxib for the management of inflammation and pain associated with breast cancer (MCF-7) for synergistic effect with anticancerous therapeutic agents. However, further in-vivo studies are required to fully characterize the anticancerous effect of pure etoricoxib and PEC stabilized etoricoxib nanosuspension.

The bright-field image of MCF-7 cells incubated with 22 μg/mL solution of drug with optimized formulation N5 is shown as Figure 7.

### 5 DISCUSSION

PEC formation was triggered by electrostatic interaction between oppositely charged polymers. The presence of positively charged ammonium ions in Ch facilitates the formation of strong coulombic bonds with the negatively charged polysaccharide. Mutual entanglement between oppositely charged polymer and simultaneous thermodynamic stability was achieved due to electrostatic interactions. The
interaction between oppositely charged ions may rise to configurational changes. Hydrophobic interaction and Van der walls force also have a significant role in the stability of the complex, specifically in case of the long branched structure as in case of used polysaccharides that is, Ch and NGP.

Ch solution acts as a host polymer and NGP solution (guest polymer) was added with continuous stirring. The excess charge of polymers, which does not participate in the formation of PEC, determines the net charge over the PEC system. This nonstoichiometric PEC was hydrophilic and hydrophilicity also depends upon molecular weight and intensity of ionizable groups. This type of hydrophilic PECs formed a colloidal dispersion and able to entrap a large number of therapeutic agents. Optimum use of a stabilizer is essential for the stabilization of nanosuspension. Stabilizer at lower concentration allows agglomeration while at higher concentration promotes Ostwald ripening. The use of a stabilizer also retards the conversion of high energy forms (low crystal packing energy) of drug nanoprecipitates into low energy forms (high crystal energy forms).

Optimized formulation was selected among nine prepared nanosuspensions based on particle size and entrapment efficiency. As discussed in different literatures smaller particles have better surface area but very small size may also result in Ostwald ripening due to very high solubility. As shown in Ostwald-Freundlich Equation (15), particle size reduction below 500 nm increases dissolution properties due to improved salvation pressure and disruption of solute-solute interaction.

\[
\log \frac{C_s}{C_\infty} = \frac{2nV}{2.303RT\sigma} 
\]

Where, \(C_s\) and \(C_\infty\) is saturated solubility and solubility of solid consisting large particles, respectively, \(\nu\) is the molar volume of

**FIGURE 7** Bright field image of A, untreated MCF-7 cells and MCF-7 cells incubated with 2 µg/mL solution of B, pure drug, and C, NS after 48 hours
particles, $R$ is gas constant, $T$ is absolute temperature, $r$ is the radius of particles, and $\rho$ is the density of particles.

Biodistribution, cellular uptake and internalization of nanosuspension depend upon the size of nanoparticles. Smaller size nanoparticles can easily enter through membrane pores but larger particles are taken up by reticuloendothelial systems. As shown by Saltin et al cellular uptake and internalization of 30 nm sized carbon nanotubes were higher than 50 nm sized carbon nanotubes. They also showed that the particulate system <200 nm shows better penetration than >500 nm sized nanoparticles. It shows that the therapeutic effect mainly depends upon the size of nanoparticles. In the present experiment, N5 showed the lowest size and good entrapment efficiency, so selected as an optimized formulation.

PEC adsorption prevents agglomeration of particles by reducing surface free energy. In the present research “bottom up” process that is, antisolvent precipitation method was used to prepare nanoparticles. Antisolvent precipitation also alters the physical properties, morphology, and size distribution of drug nanoparticles. The addition of a drug solution into antisolvent reduces the solubility of API in the system and induces rapid crystallization. Prepared nanoparticles might be crystalline or amorphous depending upon experimental conditions. Poor micromixing creates accidental zones of large number of particles that promote agglomeration. To avoid this solvent- the antisolvent system was stirred which allow uniform distribution of the supersaturation zone. Stirring speed intensifies the mixing at the molecular level and reduces the overall size of particles.

Ultrasonication also reduces nucleation induction time and width of the metastable zone. In this research homogeneous nucleation takes place as a driving force is only chemical nature (solvent-antisolvent properties). To avoid heterogeneous nucleation foreign particles must not be present in the antisolvent thus HPLC grade water was used as antisolvent in the experiment.

5.1 | Nucleation

Supersaturated solution of API has high free energy and becomes unstabilize. For the stabilization of the system, crystal formation takes place simultaneously resulting in the minimization of free energy. Free monomer “M” is formed diffuses, met each other leads to dimer “$M_2$” formation. Dimmers can form trimers “$M_3$” or dissolve to form monomer “M.” It is a bidirectional process and can be expressed as Equation (16).

$$\text{M}_n + M \xrightarrow{\text{Simultaneous process}} \text{M}_n + 1 \quad (16)$$

The size of crystals formed during the nucleation process plays a crucial role in crystal growth. A crystal size (R) is essential below high crystal easily dissolve in the solvent system. Above this size, they continue to grow. The dissolution of the crystal is due to the thermodynamic instability of crystals.

Free energy change $\Delta G$ required to form crystals having radius “R” within the supersaturated solution can be expressed using Equation (17).

$$\Delta G = -Nc \times Kb \times T \times \ln \frac{c}{C_0} + 4\pi R \times \rho y \quad (17)$$

Where $Nc$ is the number of aggregating molecules, $Kb$ is Boltzmann constant, $T$ is the temperature of the system in Kelvin, $C$ is the molar concentration of API, $C_0$ is intrinsic solubility, and $y$ is interfacial tension between solution and crystal.

The number of monomer aggregating can be calculated by following Equations (18) and (19).

$$n_e = \frac{4\pi R^3}{3\nu} \quad (18)$$

$$V = \frac{V_m}{N_a} \quad (19)$$

Where $V_m$ is the molar volume of API and $N_a$ is Avogadro’s Number.

High surface free energy at the interface of the crystal increases with interfacial tension. A crystal having high surface energy due to interfacial tension required to stabilize itself by agglomeration. Surface free energy hence surfaces tension can be reduced by the addition of stabilizer. In the present research, NGP-chitosan PEC was used as a stabilizer. NGP itself has been used to reduce surface energy to stabilize emulsion.16

5.2 | Shape and size of nanoparticles

Nanocrystals are crystalline nanoparticles and have improved saturation solubility. Crystalline nanoparticles have better dissolution characteristics due to the large surface area. Mathematically effect of nanoparticle size over dissolution rate can be expressed by using Nernst-Brunner Equation (20) (a combined form of Noyes-Whitney equation and Fick’s second law).

$$\frac{dC}{dt} = \frac{DS}{Vh} (C_s - C) \quad (20)$$

Where, $\frac{dC}{dt}$ is the rate of dissolution, $D$ is the average diffusion coefficient, $S$ is the surface area of solid particles, $D$ is the average diffusion coefficient, $S$ is the surface area of solid particles, $C_s$ is the concentration of drug in bulk, $C$ is the concentration of drug in the diffusion layer, $h$ is the thickness of the diffusion layer and $V$ is the volume of the medium. As shown in Nernst-Brunner Equation (19), an increase in saturation solubility drastically improves the dissolution rate. In another study, it was found that the Kelvin equation shows that smaller curvature of crystalline nanoparticles increases saturation solubility hence dissolution rate and therapeutic effect.5
The shape of nanoparticles affects circulation time, biodistribution, cellular residency and receptor-mediated endocytosis. Spherical nanoparticles, due to curvature, allows limited ligand binding site to interact with cellular receptors, while elongated particles facilitate better and multivalant interaction with cells. Dasgupta et al showed that elongated particles have a better aspect ratio than spherical particles and taken by cells effectively. Exocytosis of hexagonal and pentagonal particles is also lower than spherical particles. Due to the high aspect ratio, these hexagonal and pentagonal nanoparticles show a larger extent and faster rate of absorption than lower aspect ratio carrying spherical nanoparticles.

Adsorption of stabilizer that is, PEC over polymer provides slower surface integration and influences the shape of particles. Polymer adsorption only prevents crystal growth but not the rate of nucleation because nucleus clusters are very small and no stabilizer adsorption takes place at this size range.

Nucleation time plays a significant role in the overall size of nanoparticles. Longer nucleation time leads to the formation of larger irregular particles. To avoid this drug solution was added in the preheated polymer solution (45 ± 2 °C) that results in spontaneous evaporation of solvent (acetone) and simultaneous nuclei formation. The rate of mixing of solvent to antisolvent also influences the size of nanoprecipitates. Faster mixing results in a shorter time for crystal growth and allowing smaller particles.

The ratio of cationic and anionic polymers and nucleation time are the key factors that determine the size and shape of nanoparticles. Literature shows that the crystal form of drugs is physically and chemically more stable than their amorphous form. Crystalline natures of nanoparticles also prevent agglomeration and crystal growth. PEC formulation also changes polymeric orientation and molecular architecture.

Generally, the solvent antisolvent precipitation method is used to prepare amorphous nano drugs. It has been found that the antisolvent precipitation technique was utilized by a pharmaceutical manufacturer "Soliqua" to prepare amorphous nano drugs. So, in the present investigation solvent- antisolvent method was selected for the preparation of nanoparticles. The literature showed that nanodrugs such as Avinza, Emend, Focaline, Megace ES, Rapamune, and Tricor are commercialized and available in crystalline form. Chew et al also reported that the crystalline form of nano-drugs is more stable than amorphous form and preferred by the pharmaceutical manufacturer. So, based on literature, the investigator preferred the fabrication of crystalline nanoparticles.

At lower temperature, the solubility of API decreases in the solvent-antisolvent system and growth kinetics decreases that further leads to the formation of smaller particles. Submicrometer nanoparticles have more surface area as compared to a microparticulate system hence provide better dissolution profile and cellular uptake. In literature, it has been found that hexagonal nanoparticles were more effective than rod or plate-shaped nanoparticles against Staphylococcus aureus and Escherichia coli. Present research reports a new technique for the preparation of pentagonal and hexagonal nanoparticles. Properties of nanoformulations are dependent upon size as well as the shape of nanoparticles. Highly faced nanostructures have different optical and electrical properties. The fabrication of novel hexagonal nanocarriers for dissolution rate enhancement of poorly water-soluble drug was successfully achieved. Self-assembled pentagonal and hexagonal nanoparticulate system provides peculiar characteristics as a drug delivery carrier. Small traces of cubosomes were also found as byproducts. Hexagonal and pentagonal nanoparticles have the highest aspect ratio and excellent mechanical strength. They have remarkable stability. The polymeric chain length of polymers also a key factor for the size of prepared nanoparticles. The shape of nanoparticles alters internalization, biodistribution, hydrodynamic behavior in blood vessels, cellular adhesion, deposition, clearance by macrophages and cellular intake. The geometry of nanoparticles significantly alters the interaction with the target and brings notable differences in therapeutic effect. Solid tumors exhibit vascular pores cut-offs between 380 to 780 nm. So the size of nanoparticles plays a crucial role in passive tumor targeting.

5.3 | Effect of ultrasonication

Ultrasonication induces cavitations into the system and reduces the chance of agglomeration. In a study, Lanare et al showed that ultrasonication promotes uniform distribution of nanoparticles and prevents agglomeration. Ultrasound radiation assisted smaller and uniform size particles. Ultrasound rays create negative pressure zones when propagated in the medium. The negative pressure zone initiates cavitations followed by the formation of a localized hot spot. Formed hot spot further releases powerful shock waves and promotes crystal growth. Movement of shock waves from one region to another region also influences the growth rate of specific crystal faces and alters the crystal habit hence crystal lattice. Ultrasonication also causes a reduction in particle size due to abrasion at the surface of particles.

5.4 | Dissolution, drug release, and kinetic study of drug release

Solubility of API depends upon the lattice structure of molecules, which is associated with intermolecular bonds and arrangement of molecules and number of interaction points between the molecules in a crystal lattice. Any formulation strategy that changes crystal lattice due to change in crystallinity, crystallization, or salt formation may improve significantly the dissolution profile of API. Hansen proposed that drugs become soluble in the solvent when their solubility parameters are equal or the same. Total solubility parameters (δt)2 can be expressed using Equation (21).

\[ δt^2 = δd^2 + δp^2 + δn^2 \]  

where δd2, δp2, and δn2 expressed partial dispersive contribution, polar contribution, and contribution of the hydrogen bond, respectively. The presence of PEC coating and further dissociation into aqueous medium improves the contribution of hydrogen bonding and overall solubility parameters.

Strong electrostatic bonds control drug release. Crosslinking density also modulates drug release from PEC stabilized nanoparticles. An
increase in crosslinking density restricts the mobility of solvent into nanoparticles and retard drug release. Crosslinking density provides strength by reducing chain mobility and pH sensitivity. The solubilization of ionic groups into the surrounding medium leads to the formation of the aqueous channel. The rate of transport of API depends upon the pore size of the aqueous channel and size can be formed by altering the ratio of cationic and anionic polymer ratio. Improved dissolution of drugs, results in the use of a lower dose of the drug in the formulation.

Baker-Lonsdale kinetic model is derived from Higuchi kinetic model and used to describe drug release from the microsphere and nanoparticles. Based on size this model is also supported. Drug release from Baker-Lonsdale model assumes that: (i) initial drug concentration in the matrix is much higher than the solubility of drug, (ii) drug particles encapsulated in PEC coating is much smaller than thickness of system, (iii) drug particles were coated with PEC and no drug was on the surface, and (iv) drug diffusivity was constant.

Baker-Lonsdale model predicts that drug release occurs through pore formation in the system. It shows that hydrophilicity PEC solubilizes and subsequent drug release through pores. Baker-Lonsdale model was followed by all the formulations either tomato membrane or egg membrane was used as a rate-controlling biological barrier. It also shows the similar effect of both membranes as a biological barrier.

5.5 | Stability concern

PEC precipitation stabilizes drug nanoparticles by electrostatic stabilization and steric stabilization. PEC precipitation prevents close interaction between nanoprecipitates. Precipitation of prepared nanosuspension is a critical issue for pharmaceutical scientists. According to Stoke’s law (Equation (22)), particle size and viscosity directly affect the sedimentation of nanoparticles in suspension.

\[
R = \frac{2r^2(p_1 - p_2)g}{9\eta n}
\]  

(22)

Where \( R \) is sedimentation rate, \( r \) is particle size, \( p_1 \) is the density of particles, \( p_2 \) is the density of continuous phase, \( g \) is gravitational constant and \( \eta \) is the viscosity of continuous phase.

The higher viscosity of the continuous phase may create a problem at the time of dose measurement for administration.

As shown in Table 6, all the formulations show an increase in particle size except N6 and N8. Nanoparticles are tiny particles, which may be dissolved in solvent and transfer from the bulk medium to solute particles (other nanoparticles). The process of movement of dissolved solute to the surface of nanoparticles results in the crystal growth. Formulation N2, N3, N5, N6, N7, and N9 shows a very significant growth in particle size after 45 days. Formulation N8 showed lower particle size after 45 days, it may be due to surface solubilization of nanoparticles with time.

Better control over MCF-7 cells by N5 may be due to better dissolution of the drug in case of formulations. The pure drug has less solubility as compared to a nano-formulated drug. Smaller nanoparticles have more surface area hence better dissolution and therapeutic activity. Lower particle size also facilitates the penetration of nanoparticles into cancerous cells.

5.6 | In-vitro cytotoxic study

Nanoparticles in the size range of 10-100 nm are potential candidates for passive tumor targeting. They are easily interacting with tumor cells and preferably accumulate. Blood capillaries have a diameter of 5 to 6 μm so the administration of smaller particles prevents embolism and capillary blockage. This is why N5 was selected as an optimized formulation among nine formulations and used in the study. As depicted in Figure 6 pure drug (ie, etoricoxib) shows maximum control over cell growth in the concentration of 2 μg/mL. The aim of the present research was also to study the therapeutic effect of the pure drug as well as the nano-formulated drug, which was successfully achieved.

6 | CONCLUSION

In the present investigation, an attempt was made to improve the aqueous solubility of etoricoxib (selective COX-2 inhibitor) by ultrasonication assisted solvent antisolvent nanoprecipitation method. Furthermore, nanoprecipitates were stabilized by Ch-NGP PEC. Utilization of particle technology (antisolvent method) successfully improves the therapeutic benefits of poorly water-soluble drug etoricoxib. Cationic-anionic polymer-based polyelectrolyte stabilized drug nanoprecipitates open an imminent area of research due to better therapeutic benefits. The present research shows the progress in the fabrication of highly faced hexagonal and pentagonal nanoparticles by stabilization of Ch-NGP PEC and utilization of ultrasonication assisted antisolvent precipitation method. Studies also predict that the utilization of egg membrane and tomato membrane does not alter drug release kinetics from formulations but significantly differ as a biological barrier (S<50). It can be concluded from the complete research that PEC stabilized etoricoxib nanoparticles shows better control over cell growth than pure etoricoxib when used against breast cancer cells line (MCF-7).

CONFLICT OF INTEREST

Authors have no competing interests.

AUTHOR CONTRIBUTIONS

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

ETHICS

This study does not involved any animals or human.
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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