Efficiency of self-assembled etoricoxib containing polyelectrolyte complex stabilized cubic nanoparticles against human cancer cells

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Abstract
Introduction: The aim of the present research was to formulate chitosan-kheri gum polyelectrolyte complex (CKGPEC) stabilized etoricoxib containing cubic nanoparticles and evaluate against various human cancer cell lines.

Methods: The novel solvent-antisolvent method was utilized for the fabrication of nanoparticles using CKGPEC as a stabilizer. ³² factorial designs were applied to investigate the effect of concentration of chitosan (Ch) and kheri gum (KG) over entrapment efficiency and size of nanoparticles. Nanoparticles were characterized and evaluated against the human breast cancer cell line (MCF7), human colon cancer cell line (HT-29), and human skin cancer cell lines (SK-MEL-2).

Results: It was observed that the concentration of Ch and KG significantly affects the entrapment efficiency and size of nanoparticles. The entrapment efficiency of nanoparticles was found in the range of 70.21 ± 0.42% (K4) to 82.77 ± 0.29% (K6) while the size was observed 79.3 nm (K5) to 490.1 nm (K1). SEM clearly showed the cubic shape of nanoparticles. All the formulations followed Baker-Lonsdale kinetic model of drug release. The utilization of the egg membrane and tomato membrane as a biological barrier was not altering the release kinetics of the drug. Prepared nanoparticles were found to be effective against various human cancer cells but the better effect was observed against SK-MEL-2 cells than MCF-7 cells, followed by HT-29 cell in in vitro conditions.

Discussion: Taken together, it can be concluded that Ch-KG PEC stabilized nanoparticles were successfully formulated and could be utilized against various human cancer. In future clinical studies could be performed for the exact determination of therapeutic potential.

KEYWORDS
chitosan, drug delivery, etoricoxib, human cancer cell line, kheri gum, nanoparticle, polyelectrolyte

INTRODUCTION

The demand for new active moiety is increased continuously for the better therapeutic effect. Despite having good therapeutic effect, many active pharmaceutical ingredients suffer from poor aqueous solubility. Poor aqueous solubility is a major obstacle for the translation of newly developed active moiety. Popular solubility enhancement method such as solid dispersion, prodrug and salt formation leads to...
dose escalation, high cost, and higher excipient percentage in the formulation. To overcome these limitations, the solvent-antisolvent method is widely employed for the generation of drug nanocrystals. The development of nanoformulation for poorly water-soluble molecules is an extensively used strategy to improve the dissolution properties of the drug in the last decades. Solvent-antisolvent method has been touted to be a very promising bottom-up method for the preparation of nanoparticles, especially to improve the dissolution profile of less water-soluble drug candidates.2

Attempts have been made to overcome drug-associated lower solubility. The application of nanotechnology in solubility improvement gains good attention due to rich expectations of novel outcomes with procedural modification. Method attracts worldwide researchers due to their simplicity and versatility.3 Strategic improvement in nanosizing leads to improved dissolution rate hence bioavailability. Studies have shown that cyclooxygenase-2 (COX-2) is involved in tumor growth and advancement. Selective COX-2 inhibitors block tumor development through many mechanisms, in particular through antiangiogenic and proapoptotic impacts.4 In a study, Alhakamy et al discussed that an antifungal drug itraconazole shows anticancerous by same mechanism.5 Wong et al showed that NSAIDS, specially COX-2 inhibitors (celecoxib, etoricoxib) have potential anticancerous activity against various cancer types.6 Etoricoxib have pyrazole as a basic moiety and show various pharmacological activities in which anticancer activity is also involved.7 Cyclooxygenase-2 (COX-2), an inducible prostaglandin G/H synthase, is overexpressed in several human cancers, including colon cancer and thus the potential ability of a selective COX-2 inhibitor, etoricoxib, is regarded in the rat model to prevent 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in the rat model.8 In the present investigation, solubility of etoricoxib was enhanced by formulating nanoparticles and prepared formulations were evaluated for anticancerous activity against breast cancer cells.

Last few decades, researchers have shown concern regarding shape dependent therapeutic effect of nanoparticles. Shape and size modification of nanoparticles is an emerging technique to achieve prerequisite characteristics. The surface volume ratio and curvature dimension of nanoparticles is a major input to modify cellular behavior. Formation of low curvature rod and cubic nanoparticles enables them for better cellular internalization, biodistribution, and achieves unmet clinical demand.

Nanoparticles with unique morphology show better properties in vitro and in vivo due to increased surface area to volume ratio. Higher surface area to volume ratio of nanoparticles enables them to dominate over the cellular barrier for drug targeting. For better therapeutic effect, it becomes essential for nanoparticles to cross cell-associated barriers. The division of cells in the tumor tissues also positively affects the uptake of nanoparticles within the cells. The internalization of nanoparticles also depends upon the shape of formulated particles. Elongated particles provide more binding sites to interact with tumor cells receptor, hence better therapeutic effect obtained than curve shape spherical nanoparticles.9 Dasgupta et al were found that elongated particles showed a high propensity for cellular uptake than spherical particles for the same size range due to the high aspect ratio.10 Exocytosis of elongated particles also found lower than spherical particles.

Despite chitosan is superior and approved biomaterial, it lacks water solubility.11 Formation of polyelectrolyte complex between chitosan (Ch), a cationic polymer and kheri gum polysaccharide (KGP), an anionic polymer control solubility/dissolution of prepared nanoparticles in acidic (stomach), and basic (intestine) bioenvironment. Shah et al had prepared self-assembled nanoparticles by noncovalent, electrostatic interaction between oppositely charged molecules. They were also successfully utilized prepared nanoparticles for biomedical applications.12

In the present investigation, the solvent-antisolvent method occupied with ultrasonication was applied for the formulation of cubic nanoparticles stabilized with Ch-KGP polyelectrolyte complex. Furthermore, prepared formulations were evaluated for anticancerous effect against human breast cancer cell lines (MCF-7), human colon cancer cell line (HT-29), and human melanoma cell line (SK-MEL-2). To the best of our knowledge, it was first attempt to fabricate chitosan-neem gum polyelectrolyte complex stabilized nanoparticles and studied against various human cancer cell lines.

2 | MATERIAL AND METHODS

2.1 | Materials

Chitosan (molecular weight: 190 000-310 000 Da) was procured from Merck Specialties Private Limited, Mumbai, India. Ethyl alcohol and acetone were supplied by S.D. Fine Chemicals, Mumbai, India. All the chemicals were used as supplied, without any purification. In experiments, HPLC grade water was used as a solvent. Drug etoricoxib was obtained as a gift sample from Cipla Ltd, Mumbai.

As described in our previous publication, crude KGP was collected and purified using a water-based extraction process.13

2.2 | Methods

2.2.1 | Preparation of PEC stabilized nanoparticles

For the preparation of PEC stabilized nanoparticles, the antisolvent method was used. In a recent study, nonstoichiometric ratios of anionic and cationic polymers were used for the fabrication of nanoparticles as shown in Table 1.

HPLC grade water and 5% acetic acid was used as a solvent to prepare KGP and chitosan solution (20 mL each), respectively. In the solution of Ch, KGP was added dropwise and stirred at 50 rpm and 45°C for 30 minutes (PEC solution). Drug solution (10 μg/mL) was prepared by using aceton as a solvent and transferred dropwise into PEC solution by using a syringe (BD Emerald 5 mL). Moreover, the solution was stirred at 45°C for 30 minutes followed by cooling up to 35°C. Prepared formulations were stored in an airtight glass container.
2.2.2 | Factorial design

PEC stabilized nanoparticles were prepared using 3² factorial design. In the present research, the concentration of Ch and KGP was selected as independent variables while particle size and entrapment efficiency of nanoparticles were selected 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 12/06/2018) and shown in Table 2.

2.2.3 | Characterization of nanoparticles

Prepared polyelectrolyte complex stabilized nanoparticles were characterized for the following parameters:

Particle size and zeta potential analysis
Fabricated nanosuspensions were diluted to prepare 1% w/v suspension using water, the size and zeta potential was determined using zeta seizer (Malvern Instrument, version 6.32, Model No. ZEN3500, United Kingdom).

SEM analysis
Zeis EVO analyzer was used to study the morphology of nanoparticles.

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):

\[
\text{Drug loading (\%)} = \left( \frac{\text{Amount of drug found in the nanoparticles}}{\text{Amount of nanoparticles}} \right) \times 100. \tag{1}
\]

In vitro drug release
USP Type I (Rotator basket) dissolution apparatus (Lab India Dissolution Tester: DS 8000+, India) was employed to study the in vitro drug release of formulations. Tomato and egg membrane was used as a biological membrane to enclose drug samples. Drug release was carried out in 0.1 N HCl for 120 minutes followed by a study in phosphate buffer pH 7.4. The rotation of basket was kept at 50 ± 2 rpm and the medium temperature was maintained at 37 ± 0.5°C. Aliquots were withdrawn at predetermined time intervals, filtered by the help of Whatman filter paper (pore size 20 nm) and concentration was determined using a UV spectrophotometer.

Preparation of egg membrane. To prepare the egg membrane egg of chicken (Gallus gallus) was taken. Further, egg yolk was removed by an orifice made at one end of the egg. A beaker was taken, filled with acidified water and egg was kept in it. The temperature was increased

| Formulation | Independent variables |
|-------------|-----------------------|
|             | Ch (%w/v) | KGP (%w/v) |
| K1          | 0.03      | 0.03       |
| K2          | 0.03      | 0.01       |
| K3          | 0.03      | 0.02       |
| K4          | 0.01      | 0.03       |
| K5          | 0.01      | 0.01       |
| K6          | 0.01      | 0.02       |
| K7          | 0.02      | 0.03       |
| K8          | 0.02      | 0.01       |
| K9          | 0.02      | 0.02       |

| Formulation | Characterization parameters |
|-------------|-----------------------------|
|             | Physical appearance | Particle size (nm) | Zeta seizer | SEM | PDI | Zeta potential (mV) | Entrapment efficiency (%) |
| K1          | Clear solution       | 490.1             | –           | 0.317 | 34.3 | 82.46 ± 0.28 |
| K2          | Clear solution       | 310.1             | –           | 0.336 | 33.9 | 79.71 ± 0.51 |
| K3          | Clear solution       | 390.0             | –           | 0.362 | 33.6 | 75.26 ± 0.32 |
| K4          | Clear solution       | 202.0             | –           | 0.333 | 32.9 | 70.21 ± 0.42 |
| K5          | Clear solution       | 793               | 64-67       | 0.376 | 34.4 | 71.01 ± 0.36 |
| K6          | Clear solution       | 225.2             | –           | 0.382 | 33.3 | 82.77 ± 0.29 |
| K7          | Clear solution       | 189.3             | –           | 0.378 | 33.6 | 81.27 ± 0.23 |
| K8          | Clear solution       | 142.1             | –           | 0.332 | 34.9 | 76.83 ± 0.48 |
| K9          | Clear solution       | 98.3              | –           | 0.323 | 33.5 | 73.44 ± 0.46 |
up to 45 ± 2°C which causes bubbles formation. After some time, the formation of bubbles gets stops and the foam gets vanished. The egg-shell contains calcium carbonate, when it comes to the contact of acidified water it released as foam and after that only membrane was left in the beaker. The membrane was collected, washed with water and utilized as a biological membrane for drug release study.

Preparation of tomato membrane. Tomato (Solanum lycopersicum) was taken and washed with double distilled water to remove any dirt. To release the pulp a cross was made at the top of the fruit and transferred into a beaker. The temperature of the beaker was increased up to 45 ± 2°C and after 20 minutes membrane was carefully removed, washed with double distilled water and utilized for drug release study as a biological barrier.

Kinetics of drug release
The release pattern of the drug was also determined by using various kinetic models. In the present research model dependent methods (viz zero-order, first-order, higuchi model, Karsemeyer-Pepass model, Hixon-Crowel model, and Baker-Lonsdale model) and model-independent methods (viz the similarity factor determination) were applied to characterize drug release pattern.

As discussed in our previous study 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.

In the present investigation, dissolution profiles of optimized formulation, K5 was compared when drug release was performed through two different biological barriers, that is, egg membrane and tomato membrane was 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 2)

$$f_1 = \left[ \frac{\sum_{i=1}^{n} (R_i - T_i)}{\sum_{i=1}^{n} R_i} \right] \times 100,$$

where $n$ is the number of time points, $R_i$ is the dissolution value of K5 when the egg membrane was used as a biological barrier at time $t$, and $T_i$ is dissolution value of K5 when the 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 3).

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

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 a barrier.

Particle size growth analysis
In the present study, nanoparticles were formulated which were dispersed in an aqueous medium. As they are in direct contact with the aqueous medium; so, it becomes necessary to evaluate the effect of the presence of solvent in crystal growth. 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 analyzer.

Cytotoxicity screening
In vitro cytotoxicity study of optimized formulation K5 was carried out against human breast cancer cell lines (MCF-7), human colon cancer cell line (HT-29), Human melanoma cell line (SK-MEL-2) at Anti-Cancer Drug Screening Facility (ACDSF) at ACTREC, Tata Memorial Centre, Navi Mumbai. In the experiment, SRB (sulphorhodamine B) was utilized for the anticancerous activity.

3 | RESULT AND DISCUSSION
Oppositely charged polymers interact with each other, which leads to the formation of a polyelectrolyte complex. Ammonium groups of chitosan become ionize in acidic medium to form an anionic cloud; it forms electrostatic bonds when interacting with negatively charged KGP. The interaction between oppositely charged ions leads to configurational changes. Van der walls force and hydrophobic interactions also play a significant role in the stability of the complex. The use of aqueous solvent eliminates the chance of organic solvent associated with toxicity.

The characterization parameters of KGP-Ch PEC stabilized nanoparticles were shown in Table 2.

Generally, nonaqueous solvents are used to improve the solubility of poorly water-soluble drugs. The formation of nanoparticles results in the improved dissolution characteristics of poorly water-soluble BCS Class II drug etoricoxib. In the present experiment, KGP solution was added into the chitosan solution to form polyelectrolyte and excess charge that was not precipitated to form PEC determined the overall charge of the system. This nonstoichiometric PEC was hydrophilic in nature and its hydrophilicity depends upon molecular weight and intensity of ionizable groups.

Chitosan showed worm-like conformation in dilute solution while KGP showed coiled conformation. When the solution of KGP (guest solution) was added in the chitosan solution (host solution), polyelectrolyte complex formation takes place as colloidal dispersion.

Table 3 shows formulating parameters and results of optimized batch K5.

The reduction of particle size, due to the formation of nanoprecipitate leads to a higher value of Gibb’s free energy. To lower down the value of Gibb’s energy, suspended particles start to form agglomerate. Utilization of suitable hydrophilic stabilizer lower down Gibb’s energy and prevent crystal growth. 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).
Gibb’s free energy ($\Delta G$) for the cubic system can be expressed as Equation (4).

$$\Delta G = \gamma A_{\text{cube}} - \Delta \gamma V_{\text{cube}}.$$  \hspace{1cm} (4)

Equation (4) can be solved as Equation (5).

$$\Delta G = \gamma 6r^2 - \Delta \mu r^3.$$  \hspace{1cm} (5)

where $\gamma$ is the surface energy of a solute-solvent system, $\Delta \mu$ is chemical potential, $A_{\text{cube}}$ is of cubic particle and $V_{\text{cube}}$ is the volume of cubic particle, and $r$ is the width of the cubic particle.

The antisolvent method of nanoparticle preparation is effective in the case of heat-sensitive API as it is carried out near to ambient temperature and pressure. It demands relatively lesser energy and low-cost equipment compared to other methods of nanoparticle preparation. The antisolvent method is scalable as compared to the bottom-up process. The antisolvent method of nanoparticle preparation easily modifies the solubility and shape of particles but has a significantly low effect on crystallization kinetics. The present research was not included any toxic solvent or component so environmentally friendly in nature.

3.1 | Factorial design

In the present investigation, $3^2$ full factorial design was employed to evaluate the effect of concentration of cationic polymer (Ch) and concentration of anionic polymer (KGP) (independent variables) on particle size and entrapment efficiency (dependent variable). The reduced equation to measure the response (particle size and entrapment efficiency) having statistical significance for $3^2$ factorial design can be shown as below Equation (6).

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2.$$  \hspace{1cm} (6)

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 KGP, respectively.

From the experimental data, Equation (6) was solved to generate the effect of concentration of Ch and KGP over particle size (Equation 7) and entrapment efficiency (Equation 8) of PEC stabilized nanoparticles.

$$\text{Particle size (nm)} = 236.27 + 75.97 (X_1) + 38.88 (X_2) + 6.37 (X_1X_2) + 31.01 (X_{12}) - 0.52 (X_{22}).$$  \hspace{1cm} (7)

$$\text{Entrapment efficiency (%) } = 76.99 + 1.49 (X_1) + 0.71 (X_2) + 0.39 (X_1X_2) - 0.06 (X_{12}) - 0.05 (X_{22}).$$  \hspace{1cm} (8)

Surface plot for effect of independent variables over size and entrapment efficiency of nanoparticles was depicted by Figures 1 and 2, respectively.

Various characterization parameters of prepared nanoparticles were shown in Table 2. Among all the formulations, K5 (concentration of Ch 0.01%w/v, concentration of KGP 0.01%w/v) shows lowest particle size, that is, 79.3 nm. So K5 was considered as optimized formulations.

Birch et al have been synthesized the self-assembled polyelectrolyte complex nanoparticles by using chitosan and pectin.
showed that polymer concentration has the significant effects on the characteristics of nanoparticles.  

3.2 | Nucleation

Nucleation without the presence of foreign particles is known as homogeneous nucleation and can be expressed as Equation (9).

\[
B^0 = A_{\text{hom}} \exp \left( -\frac{16 \pi \sigma^3 \nu^2}{3 k T^3 \ln(1 + S)^2} \right),
\]

where \( B^0 \) is the rate of nucleation, \( A_{\text{hom}} \) is pre-exponential factor, \( \gamma \) is interfacial tension at solid particles and solvent system, \( \nu \) is molar volume, and \( T \) is the absolute temperature.

It can be concluded from above Equation (8), that the rate of nucleation increases with interfacial energy. High nucleation rate leads to precipitation of solute particles and in this case, supersaturation is utilized mainly for nucleation, not for the growth of particles. To achieve a narrow size distribution of polyelectrolyte stabilized etoricoxib nanoparticles, researchers create a high degree of supersaturation, uniform distribution of antisolvent (by mixing) and controlled the growth of nanoparticles.

The formation of nanoparticles using antisolvent-solvent methods involves the formation of saturation zone, nuclei generation and particle growth in a successive manner. The initiation of particle formation depends upon the characteristics of the supersaturation zone in the solution. The size, shape and morphology of the nucleus depend upon supersaturation conditions. Mathematically supersaturation of API insolvent can be expressed using Equation (10)

\[
S = \frac{C}{C^*},
\]

where \( C \) and \( C^* \) are an actual concentration of API in solution and equilibrium solubility of API in solvent-antisolvent system, respectively.

The high degree of supersaturation leads to a higher nucleation rate due to lower Gibb's free energy. Mathematically nucleation rate \((B^0)\) can be expressed as Equation (11).

\[
B^0 = A_{\text{hom}} \exp \left( -\frac{16 \pi \sigma^3 \nu^2}{3 k T^3 \ln(1 + S)^2} \right),
\]

where \( A_{\text{hom}} \) depends upon the growing mechanism of solute particles, \( k \) is Boltzmann constant, \( \gamma \) is interfacial tension, and \( T \) is the absolute temperature. Equation (10) easily elicits that the rate of nucleation mainly depends upon interfacial energy and supersaturation. The rate of nucleation also depends upon the characteristics of the metastable zone. A metastable zone is a region where no crystallization takes place within a time frame. To achieve better nucleation, the width of the metastable zone should be very narrow. Induction time is the lag time between supersaturation and nucleation and it is another parameter used to characterize nucleation rate.

Uniform and a high degree of supersaturation lead to the formation of lower size range nanoparticles with narrow size distribution. Higher nucleation rates lead to the formation of nanoparticles and in such cases, particle growth inhibited due to consumption by nucleation. Nucleation and particle growths are a simultaneous process and both target for supersaturation area. Controlled mixing causes coprecipitation of etoricoxib with polyelectrolyte, results in the formation of nanosuspension.

3.3 | Role of stabilizer

Formation of the drug nucleus in the antisolvent system leads to the increased surface area to volume ratio. These formed nuclei have high surface energy thus thermodynamically unstable. Formed nuclei start to form agglomerate to minimize surface energy and stabilize them.

Precipitated nanorange nucleus also becomes suitable candidates for Ostwald ripening. To avoid destabilization of precipitated nuclei either by agglomeration, crystallization, or Ostwald ripening two approaches have been used. The first and most widely used method is the utilization of a stabilizer to coat over initially precipitated API nuclei. A second approach is a kinetic approach. The kinetic approach uses high-energy input into the solvent-antisolvent system. Energy can be supplied by ultrasonication or high-speed homogenization. This transferred energy increases the kinetic energy of precipitated nuclei and prevents particle agglomeration. In the present investigation, KGP-Chitosan was used as a stabilizer. They formed polyelectrolyte complex over precipitated nuclei and stabilize them. Polyelectrolyte complex formation and subsequent irradiation by ultrasonic waves.
lead to the formation of cubic shape nanoparticles (Figure 3). It has been discussed in many studies that efficient and stabilize nanoparticles are formed by the utilization of both stabilizer and ultrasound waves. So in the present research, both are used in the preparation of nanoparticles.\textsuperscript{17}

The stabilizer also increases the nucleation rate and stabilizes the nuclei by reducing interfacial energy. This results in reduce particle-particle interaction and a decrease in the overall size of precipitated particles. The stabilizer also prevents secondary nucleation, which is an undesirable phenomenon to prevent agglomeration and Ostwald ripening.

The presence of electric charge over particles due to PEC formation causes repulsion between stabilized particles and prevents aggregation.

3.4 \hspace{2em} \textbf{Shape and size of nanoparticles}

SEM study shows that etoricoxib formed acicular crystals without stabilizer when precipitated using the solvent-antisolvent method (Figure 3). Reverchon et al also discussed that in most of the cases acicular crystals are formed during API crystallization.\textsuperscript{18} They also showed that generally amorphous particles are formed during solvent-antisolvent precipitation method because the process is spontaneous and API molecules have not enough time to go through organized precipitation.

The shape of nanoparticles also influences the circulation time and dynamics, cell internalization, and transport behavior. Surface curvature depends upon both the size and shape of particles. Curvature defines the contact between nanoparticles and cell membranes. In a study, Chen et al found that spherical gold particle was easily taken by HeLa cells as compared to rod-shaped gold particles of the same size.\textsuperscript{2} The reason behind this opposite behavior is due to the difference in nanoparticle curvature.\textsuperscript{19}

Present research reports a new technique for the preparation of cubic nanoparticles. Properties of nanoformulations are dependent upon size as well as the shape of nanoparticles. Highly faced nanostructures have different optical and electrical properties.

Particle formation is governed by mixing time and precipitation time (or induction time). Damkohler number ($D_{\text{a}}$) is an arbitrary

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{SEM image of, A, pure drug precipitate, B-D, cubic nanoparticle}
\end{figure}
number used to show the effect of mixing time \( t_{\text{mixing}} \) and precipitation time \( t_{\text{precipitation}} \) over particle formation. Mathematically, Damkohler number is the ratio of \( t_{\text{mixing}} \) and \( t_{\text{precipitation}} \). When the value of \( D_a \) is greater than 1, the process is controlled by mixing. In the present study, \( t_{\text{precipitation}} \ll t_{\text{mixing}} \), hence the value of \( D_a \) is greater than 1 and the process is mixing controlled. It was also found in the literature that \( D_a > 1 \) results in the broader size distribution of nanoparticles but utilization of stabilizer results in narrow size distribution. The narrow size distribution of particle is also supported by PDI value as shown in Table 2. PDI value 0.1 to 0.25 indicates narrow size distribution while greater than 0.5 refers to broad distribution.

The shape of nanoparticles affects the circulation time, biodistribution, cellular residency and receptor-mediated endocytosis.\(^{20}\) Spherical nanoparticles, due to curvature, allows limited ligand binding site to interact with the cellular receptors, while elongated particles facilitate better and multivalent interaction with the cells. Dasgupta et al showed that elongated particles have a better aspect ratio than spherical particles and taken by cells effectively.\(^{21}\) Due to the high aspect ratio, cubic nanoparticles show a larger extent and faster rate of absorption than the lower aspect ratio carrying spherical nanoparticles. PEC formulation also changes polymeric orientation and molecular architecture.

### 3.5 Effect of ultrasonication

Ultrasound breaks any formed agglomerate. Ultrasound also controls the size, size distribution and crystal habit of formed nanoparticles. Crystal faces are influenced by cavitations and abrasion caused by ultrasound.

In a study, Dalvi and Dave observed that sonication dependent precipitation leads to the formation of diamond-shaped particles.\(^{22}\)

Ultrasound increases the formation of particles by accelerating the diffusion process that further results in the reduction in induction time. Formation of cubic shaped particles may be due to the utilization of polyelectrolyte complex as a stabilizer or due to ultrasound waves or both. Ultrasound waves convert less ordered high-energy form to more ordered low energy form. Ultrasound also helps in adsorption and reordering of stabilizer molecules at the interface. It also decreases surface energy and interface and improved the stability of nanoparticles.

### 3.6 Dissolution and drug release

Hydrophilicity of the formulation determines the interaction of the drug with a biological fluid. PEC coating permits the ease of ionization and solubilization into an aqueous medium that is essential for therapeutic effect. Polyelectrolyte also improves the contribution of hydrogen bonding and overall solubility parameters. When nanoparticles come into the contact of biological fluids, ionization and simultaneous solubilization of ionic groups take place that further leads to the formation of an aqueous channel. The size of the formed pore depends upon the ratio of cationic and anionic polymer, which determines the movement of API toward the surroundings. Improved dissolution of drugs results in the use of a lower dose of the drug and reduces the dose-dependent toxicity of the drug.

Drug release data shows a peculiar drug release pattern of nanoparticles. All the formulation shows three phases of drug release viz initial immediate release followed by sustained release and finally the burst release. Time of 80% of drug release \( T_{80} \) from the formulation was summarized in Table 4.

Minimum and maximum \( T_{80} \) were found for K4 and K2, respectively, when the egg membrane was used as a biological membrane. Minimum and maximum \( T_{80} \) were found for K8 and K4, respectively, when the tomato membrane was used as a biological membrane.

Time-dependent drug release behavior of formulations, when the egg membrane and tomato membrane were utilized as biological barrier data were shown in Tables 5 and 6, and Figures 4 and 5.

K5 showed \( 59.17 \pm 1.75\% \) drug release in 45 minutes, while \( 99.98 \pm 7.37\% \) drug release was found after 7 hours when the egg membrane was used as a biological barrier. When the tomato membrane was utilized as a biological barrier, K5 showed \( 56.35 \pm 2.43\% \) drug release in 45 minutes, while \( 99.93 \pm 5.36\% \) drug release was observed after 7 hours. It can be concluded from drug release data that utilization of egg membrane and tomato membrane were not significantly changed the drug release pattern from the formulation and summarized in Tables 5 and 6.

The kinetic model study is an essential parameter to predict the release characteristics of the drug from formulation to external medium. Data obtained after kinetic studies of drug release (Ch-KGP) were shown in the supplementary material. Drug release from Baker-Lonsdale’s model assumes that: (a) initial drug concentration in the matrix is much higher than solubility of drug, (b) drug particles encapsulated in PEC coating is much smaller than thickness of system, (c) drug particles were coated with PEC and no drug was on the surface, and (d) drug diffusivity was constant.

Baker-Lonsdale’s model of drug release can be expressed as Equation (12).

| Formulation | Egg membrane as biological barrier | Tomato membrane as biological barrier |
|-------------|-----------------------------------|---------------------------------------|
| K1          | 152 ± 4.54                        | 329 ± 2.64                            |
| K2          | 181 ± 5.45                        | 135 ± 3.65                            |
| K3          | 180 ± 3.63                        | 373 ± 3.64                            |
| K4          | 132 ± 4.68                        | 376 ± 2.75                            |
| K5          | 178 ± 3.63                        | 124 ± 3.64                            |
| K6          | 135 ± 2.68                        | 135 ± 4.77                            |
| K7          | 181 ± 4.53                        | 124 ± 5.33                            |
| K8          | 175 ± 3.57                        | 90 ± 3.67                             |
| K9          | 152 ± 4.62                        | 210 ± 5.73                            |
TABLE 5  Time-dependent drug release study of Ch-KGP
Polyelectrolyte complex stabilized nanoparticles through the egg membrane

| Batch | Cumulative % drug release (in 45 min) | Cumulative % drug release (in 7 h) | Actual % drug release (between 45 min and 7 h) |
|-------|--------------------------------------|-----------------------------------|-----------------------------------------------|
| K1    | 65.74 ± 1.53                         | 99.91 ± 6.87                      | 34.08 ± 5.64                                  |
| K2    | 62.61 ± 0.93                         | 99.98 ± 7.57                      | 37.23 ± 6.74                                  |
| K3    | 62.54 ± 1.03                         | 99.97 ± 7.29                      | 37.33 ± 5.75                                  |
| K4    | 55.76 ± 2.74                         | 99.98 ± 6.33                      | 44.04 ± 5.64                                  |
| K5    | 59.17 ± 1.75                         | 99.98 ± 7.37                      | 40.36 ± 6.36                                  |
| K6    | 54.83 ± 2.04                         | 99.94 ± 5.85                      | 45.06 ± 4.82                                  |
| K7    | 54.97 ± 1.68                         | 99.97 ± 6.37                      | 45.11 ± 5.78                                  |
| K8    | 56.02 ± 1.74                         | 99.98 ± 8.27                      | 43.58 ± 4.76                                  |
| K9    | 56.73 ± 2.02                         | 99.96 ± 6.26                      | 43.01 ± 7.37                                  |

TABLE 6  Time-dependent drug release study of Ch-KGP PEC stabilized nanoparticles through tomato membrane

| Batch | Cumulative % drug release (in 45 min) | Cumulative % drug release (in 7 h) | Actual % drug release (between 45 min and 7 h) |
|-------|--------------------------------------|-----------------------------------|-----------------------------------------------|
| K1    | 47.95 ± 1.75                         | 98.72 ± 5.75                      | 50.63 ± 6.74                                  |
| K2    | 38.79 ± 2.57                         | 99.18 ± 6.36                      | 60.15 ± 6.68                                  |
| K3    | 31.94 ± 1.75                         | 97.47 ± 6.83                      | 65.38 ± 5.86                                  |
| K4    | 41.69 ± 2.98                         | 96.89 ± 4.85                      | 55.02 ± 7.49                                  |
| K5    | 56.35 ± 2.43                         | 99.93 ± 5.36                      | 43.36 ± 6.86                                  |
| K6    | 47.50 ± 2.50                         | 99.94 ± 4.65                      | 47.22 ± 7.48                                  |
| K7    | 52.26 ± 2.57                         | 99.98 ± 5.51                      | 47.49 ± 7.80                                  |
| K8    | 74.42 ± 1.67                         | 98.68 ± 4.27                      | 35.51 ± 5.96                                  |
| K9    | 51.12 ± 2.69                         | 99.04 ± 4.85                      | 47.84 ± 4.82                                  |

\[ f_1 = \frac{3}{2} \left[ 1 - \left( 1 - \frac{M_1}{M_{\infty}} \right)^{\frac{1}{g}} \right] \frac{M_1}{M_{\infty}} = k_1 t, \]  

where, the release rate constant, \( k_1 \), corresponds to the slope.

Baker-Lonsdale model is derived from the Higuchi kinetic model of drug release. It describes that drug release occurs through pores. The Baker-Lonsdale model predicts drug release through nanoformulation or microformulations which are spherical or about the spherical in morphology. SEM image (Figure 3) also shown that the length of particles is close to width and nanoparticles are almost spherical in morphology. Baker-Lonsdale model is also supported by the size and morphology of formulations. Bhatia et al have been developed a carrier system in the form of poly electrolyte complex of the amphotericin B by using chitosan and porphyran as oppositely charged polymers and tripolyphosphate as a crosslinking agent. The kinetics of drug release was evaluated by using various mathematical models and the Baker-Lonsdale was the best fit model for the developed formulation. Data obtained in that investigation, also showed that polyelectrolyte complex based formulations followed Baker-Lonsdale kinetic model of drug release and support the kinetic model of drug release obtained in present polyelectrolyte complex based formulations.23

Quadrado et al have been prepared microparticles based on carboxymethyl starch/chitosan polyelectrolyte complex as vehicles for drug delivery. It was observed during study that for the simulated gastric fluid, the Baker-Lonsdale model fits better for the release of drug from spherical matrices. It also supports kinetic of drug release obtained in present investigation.24

From the data, it was also observed that the utilization of tomato membrane and egg membrane as a biological barrier; do not change the kinetics of drug release.

The model-independent approach predicts the value of difference factor \( (f_3) \) 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. For the same parameters, K5 showed 17.04 and 29.34 difference factors and similarity factors, respectively. In Table 7, drug release kinetics study of Ch-KGP stabilized nanoparticles through tomato membrane, in Table 8 drug release kinetics study of Ch-KGP stabilized nanoparticles through the egg membrane.

### 3.7 Stability concern

Particle size growth analysis of prepared formulations was carried out. In suspended conditions, the size of nanoparticles may be decreased due to the relative movement of solvent or particle-particle collision. These mechanical processes are responsible for the formation of the tiny embryo and lead to the initiation of secondary nucleation. Secondary nucleation also depends upon stirring (impeller rotation) and concentration of solute. Mathematically it can be expressed as Equation (13):

\[ B^2 = k_n W^M \Delta C^g, \]  

where \( W \) is impeller rotation speed, \( M_1 \) is the concentration of solid, \( \Delta C \) is the difference between primary and secondary nucleation.

The temperature of the medium is also responsible for the alteration of nanoparticle size. Temperature changes the solubility of nanoparticles. Effect of temperature on the crystal growth can be expressed by using Arrhenius Equation (14)

\[ G = k_G \Delta C^g, \]  

where \( G \) is the crystal growth rate, \( k_G \) is constant depends upon temperature, and \( g \) is crystal growth order.

At higher temperatures, the Brownian movement increases due to higher kinetic energy that further leads to agglomeration.
As shown in Table 4, after 45 days, the size of nanoparticles increased in all the formulations, while entrapment efficiency was decreased due to the leaching of etoricoxib from the nanoparticles.

As discussed by Yadollahi et al, minimum zeta potential required for electrostatic stabilization is ±30 mV. In the present study, all the prepared formulations showed >30 mV potential and can be considered as stabilized formulation.

Gibb’s free energy is a driving force for the conversion of amorphous drugs into crystalline ones. The presence of solvent molecules within the solute matrix facilitates solute relaxation and lattice restabilization. The stability study parameters of formulations (K1-K9) were shown in Table 9.

3.8 | In vitro cytotoxic study

As depicted in Figure 4, optimized formulation K5 showed significant control overgrowth of human breast cancer cells (MCF-7), human colon cancer cell line (HT-29) and human melanoma cell line (SK-MEL-2). As the concentration of K5 increases, it showed significantly better control overgrowth of all three human cancer cell lines. It can be concluded from Figure 6 that formulation K5 showed better control overgrowth of SK-MEL-2 cancer cells than MCF-7 cells followed by HT-29 cells. Findings of the result elicit the fact that chitosan-KGP polyelectrolyte complex stabilized etoricoxib nanoparticles can be used for the management of cancer-associated pain and inflammation for synergistic effect with anticancerous therapeutic agents. However,
Further in vivo studies are required to fully characterize the anticancerous effect of pure etoricoxib and chitosan-KGP polyelectrolyte complex stabilized etoricoxib nanosuspension.

Formulation K5 showed a better therapeutic effect due to the smaller size. APIs have size-dependent solubility. The solubility of etoricoxib is less as compared to the solubility of nanoformulated etoricoxib. Cubic shaped nanoparticles (K5) facilitate cellular penetration and leads to pronounced internalization and biodistribution. The size of nanoparticles was found to be 79.3 nm and it was observed in different studies that nanoparticles below 100 nm are potential candidates for passive tumor targeting and preferably accumulate in the tumor cells. This is why K5 was selected as an optimized formulation among nine formulations and used in the study. The bright-field image of various human cancer cell lines incubated with 2 μg/mL solution of K5 is shown in Figure 7.

Rivas et al have been prepared the polymethacrylate-alginate polyelectrolyte complex nanoparticles for the delivery of lysozyme. It was investigated that the preparation at high concentration showed cytotoxicity for the HeLa cells. Result is in accordance with present investigation.26

4 | CONCLUSION

In this study, the solvent-antisolvent method was successfully employed for the preparation of nanoparticles. Chitosan-Kheri gum polyelectrolyte complex was prepared and further utilized as a stabilizer to prepare nanoparticles. SEM images showed the cubic morphology of PEC stabilized nanoparticles. Experimentation demonstrates that the concentration of Ch and KG has a significant
### Table 7: Drug release kinetics study of Ch-KGP stabilized nanoparticles through tomato membrane

| Batch | Kinetics | Zero-order kinetics | First-order kinetics | Higuchi kinetics | Baker Lonsdale kinetics | Hixson-Crowell kinetics | Korsmeyer-Peppas kinetics |
|-------|----------|---------------------|---------------------|------------------|-------------------------|-------------------------|--------------------------|
|       | $R^2$ K0 | $R^2$ K0 | $R^2$ K0 | $R^2$ K0 | $R^2$ K0 | $R^2$ K0 | $R^2$ K0 |
| K1    | 0.420 0.044 | 0.107 0.0009 | 0.719 2.109 | 0.920 0.111 | 0.866 0.002 | 0.054 0.039 | 1.838 |
| K2    | 0.335 0.047 | 0.119 0.0011 | 0.659 1.996 | 0.957 0.076 | 0.619 0.002 | 0.126 0.086 | 2.283 |
| K3    | 0.681 0.054 | 0.187 0.0013 | 0.847 1.368 | 0.902 0.078 | 0.944 0.003 | 0.008 0.021 | 2.389 |
| K4    | 0.521 0.046 | 0.125 0.0011 | 0.801 1.850 | 0.915 0.100 | 0.941 0.002 | 0.036 0.035 | 1.982 |
| K5    | 0.661 0.212 | 0.283 0.0011 | 0.859 1.629 | 0.927 0.130 | 0.892 0.010 | 0.098 0.054 | 1.624 |
| K6    | 0.74 0.206 | 0.320 0.0059 | 0.898 1.383 | 0.951 0.100 | 0.799 0.008 | 0.052 0.047 | 1.821 |
| K7    | 0.672 0.228 | 0.307 0.0059 | 0.866 1.493 | 0.938 0.114 | 0.857 0.011 | 0.117 0.067 | 1.755 |
| K8    | 0.367 0.126 | 0.179 0.004 | 0.577 2.617 | 0.910 0.195 | 0.633 0.006 | 0.003 0.003 | 1.192 |
| K9    | 0.806 0.212 | 0.322 0.0059 | 0.922 1.375 | 0.923 0.113 | 0.880 0.009 | 0.037 0.037 | 1.748 |

### Table 8: Drug release kinetics study of Ch-KGP stabilized nanoparticles through the egg membrane

| Batch | Kinetics | Zero-order kinetics | First-order kinetics | Higuchi kinetics | Baker Lonsdale kinetics | Hixson-Crowell kinetics | Korsmeyer-Peppas kinetics |
|-------|----------|---------------------|---------------------|------------------|-------------------------|-------------------------|--------------------------|
|       | $R^2$ K0 | $R^2$ K0 | $R^2$ K0 | $R^2$ K0 | $R^2$ K0 | $R^2$ K0 | $R^2$ K0 |
| K1    | 0.241 0.036 | 0.074 0.0009 | 0.833 1.832 | 0.928 0.151 | 0.399 0.002 | 0.791 0.198 | 3.332 |
| K2    | 0.278 0.038 | 0.078 0.0009 | 0.858 1.776 | 0.936 0.143 | 0.583 0.002 | 0.838 0.202 | 3.506 |
| K3    | 0.307 0.030 | 0.077 0.0009 | 0.767 1.920 | 0.907 0.162 | 0.817 0.002 | 0.616 0.150 | 2.819 |
| K4    | 0.200 0.036 | 0.072 0.0009 | 0.857 1.775 | 0.917 0.141 | 0.344 0.002 | 0.869 0.248 | 4.078 |
| K5    | 0.274 0.038 | 0.080 0.0009 | 0.887 1.706 | 0.935 0.139 | 0.427 0.002 | 0.892 0.222 | 3.855 |
| K6    | 0.228 0.037 | 0.076 0.0009 | 0.873 1.734 | 0.916 0.139 | 0.576 0.002 | 0.882 0.244 | 4.137 |
| K7    | 0.348 0.042 | 0.095 0.0009 | 0.831 1.565 | 0.903 0.141 | 0.773 0.002 | 0.624 0.208 | 3.990 |
| K8    | 0.309 0.039 | 0.085 0.0009 | 0.864 1.677 | 0.910 0.144 | 0.743 0.002 | 0.784 0.210 | 3.784 |
| K9    | 0.255 0.037 | 0.077 0.0009 | 0.867 1.755 | 0.913 0.145 | 0.658 0.002 | 0.860 0.221 | 3.787 |

### Table 9: Stability studies parameters of formulations (K1-K9)

| Formulation | Particle size (nm) | Entrainment efficiency (%) |
|-------------|-------------------|-----------------------------|
|             | Initial day | After 45 days | Initial day | After 45 days |
| K1          | 490.1     | 493.3       | 82.46 ± 0.28 | 81.87 ± 0.17 |
| K2          | 310.1     | 312.4       | 79.71 ± 0.51 | 76.98 ± 0.22 |
| K3          | 390.0     | 392.9       | 75.26 ± 0.32 | 74.76 ± 0.49 |
| K4          | 202.0     | 205.1       | 70.21 ± 0.42 | 69.88 ± 0.19 |
| K5          | 79.3      | 82.3        | 69.01 ± 0.36 | 67.23 ± 0.28 |
| K6          | 225.2     | 227.8       | 82.77 ± 0.29 | 81.65 ± 0.41 |
| K7          | 189.3     | 191.4       | 81.27 ± 0.23 | 80.79 ± 0.39 |
| K8          | 142.1     | 145.6       | 76.83 ± 0.48 | 76.12 ± 0.33 |
| K9          | 98.3      | 101.3       | 73.44 ± 0.46 | 70.86 ± 0.31 |
effect on drug loading and the size of nanoparticles. Among all the formulations, K5 (concentration of Ch 0.01%w/v, concentration of KGP 0.01%w/v) shows lowest particle size, that is, 79.3 nm. So K5 was considered as optimized formulations. All the prepared formulations were able to release drugs up to 420 minutes. It was concluded from the release data that formulations release the drug in three phases viz initial immediate release followed by sustained release and finally the burst release. It was interesting to note that the egg membrane and tomato membrane were not changed the drug release kinetics when utilized as a biological barrier. All the formulations followed Baker-Lonsdale model of drug release; either egg membrane or tomato membrane was utilized as a biological membrane. Moreover, fabricated nanoparticles showed significant anticancerous activity against various human cancer cells viz breast cancer (MCF-7), colon cancer (HT-29), and skin cancer (SK-MEL-2). Results predict that formulation K5 showed better control overgrowth of SK-MEL-2 cancer cells than MCF-7 cells followed by HT-29 cells. Further in vivo studies are envisaged to explore potential therapeutic applications of Ch-Kg polyelectrolyte complex stabilized nanoparticles.

**FIGURE 6** In vitro anticancer effect of the K5 against human breast cancer cell lines (MCF-7), human colon cancer cell line (HT-29), human melanoma cell line (SK-MEL-2)

**FIGURE 7** Bright-field image of, A, MCF-7 cells, B, HT-29 cells, and C, SK-MEL-2 cells incubated with 2 μg/mL solution of K5 after 48 hours
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CONFLICT OF INTEREST
Authors have no conflict of interest.

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 STATEMENT
No animal activity was involved in the present research. So, Ethical approval is not required.

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