Research Article

Formulation Development And Evaluation Of A Thermo-Sensitive In-Situ Gel Incorporating Allium Sativum (Allicin) Infused Chitosan Nanoparticles Against Acanthamoeba Keratitis

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1.ABSTRACT

Acanthamoeba keratitis (AK) is a severe, vision-threatening corneal infection predominantly affecting contact lens users. Current treatment regimens require prolonged administration and are often associated with toxicity and poor patient compliance due to limited ocular bioavailability. Allicin, a bioactive sulfur-containing compound derived from garlic, exhibits potent antimicrobial and anti-amoebic activity but suffers from instability and rapid degradation under physiological conditions. The present study aimed to formulate and evaluate a thermosensitive in situ gel incorporating allicin-loaded chitosan nanoparticles for enhanced ocular delivery against Acanthamoeba keratitis. Allicin-loaded chitosan nanoparticles were prepared by ionic gelation using sodium tripolyphosphate (TPP) and characterized for particle size, zeta potential, entrapment efficiency, and morphology. The optimized nanoparticles were incorporated into a poloxamer-based thermosensitive in situ gel. The formulation was evaluated for pH, gelation temperature, viscosity, drug content, in vitro drug release, and anti-amoebic activity. The optimized formulation demonstrated nanosized particles with high entrapment efficiency, appropriate gelation at ocular temperature, and sustained drug release up to 24 hours. Enhanced anti-amoebic activity was observed compared to free allicin. The developed formulation shows potential as a promising ocular drug delivery system for the management of Acanthamoeba keratitis.

Keywords: Allicin-loaded nanoparticles, chitosan nanocarriers, thermosensitive in situ gel, ocular drug delivery, Acanthamoeba keratitis, sustained drug release, mucoadhesive ophthalmic formulation, biodegradable polymeric nanoparticles.

2.INTRODUCTION

Acanthamoeba Keratitis

Acanthamoeba keratitis is a rare but serious infection of the cornea caused by protozoa belonging to the genus Acanthamoeba. The disease is commonly associated with poor contact lens hygiene, exposure to contaminated water, or corneal trauma. Clinically, it presents with severe ocular pain, photophobia, redness, blurred vision, and ring-shaped corneal infiltrates. If not treated promptly, it may result in corneal scarring or permanent vision loss. Conventional treatment includes topical biguanides (poly hexamethylene biguanide) and diamidines (propamidine). However, treatment duration may extend for several months, and recurrence is common due to the presence of resistant cyst forms.

Allicin as a Potential Anti-amoebic Agent

Allicin is an organosulfur compound derived from the enzymatic conversion of alliin in garlic (Allium sativum). It possesses broad-spectrum antimicrobial, antifungal, antiviral, and antiparasitic properties. Studies suggest that allicin exerts its effect by reacting with thiol-containing enzymes, disrupting essential metabolic pathways in microorganisms. Despite its therapeutic potential, allicin is chemically unstable and rapidly degrades when exposed to heat, light, or physiological pH, limiting its direct ocular application.

Chitosan, a natural cationic polysaccharide obtained from chitin, is widely used in ocular drug delivery due to its biocompatibility, biodegradability, and mucoadhesive properties. Chitosan nanoparticles enhance corneal permeability and prolong drug residence time. Thermosensitive in situ gels remain liquid at room temperature

and undergo sol-to-gel transition at ocular temperature (34–37°C). Poloxamer 407-based systems are commonly used for ocular thermosensitive formulations. Combining allicin-loaded chitosan nanoparticles with a thermosensitive gel system may improve stability, enhance ocular retention, provide sustained release, and increase anti-amoebic efficacy.

3.MATERIALS AND METHODS

3.1.Materials

Allicin was used as the active anti-amoebic agent in the formulation. Chitosan (low molecular weight) was selected as the polymer for nanoparticle preparation due to its biocompatibility and mucoadhesive properties. Sodium tripolyphosphate (TPP) was used as the cross-linking agent for ionic gelation. Poloxamer 407 served as the primary thermosensitive polymer, and Poloxamer 188 was incorporated to modify gelation temperature and viscosity. Glacial acetic acid was used for dissolving chitosan, and distilled water was used as the vehicle for preparation of all formulations. Simulated tear fluid (pH 7.4) was prepared for in vitro release studies. All materials were of analytical grade and were purchased from Dhamtec Pharma and Consultants, B-204, Silver Springs, Opposite MIDC Office, Taloja, Navi Mumbai – 410208.

3.2.METHODS

3.2.1.Preparation of allicin-loaded chitosan nanoparticle

Allicin-loaded chitosan nanoparticles were prepared using the ionic gelation method. Chitosan solution (0.1–0.2% w/v) was prepared by dissolving chitosan in 1% v/v acetic acid under constant magnetic stirring. The solution was filtered to remove undissolved particles. Sodium tripolyphosphate (TPP) solution (0.1% w/v) was prepared separately in distilled water .Allicin extract was added to the chitosan solution under continuous stirring. The TPP solution was then added dropwise to the chitosan–allicin mixture under high-speed magnetic stirring. Nanoparticles were formed spontaneously due to electrostatic interaction between positively charged chitosan and negatively charged TPP. The resulting suspension was stirred for 1 hour to ensure complete crosslinking. The nanoparticles were collected by centrifugation at 15,000 rpm for 30 minutes, washed with distilled water, and lyophilized for further studies.

3.2.2.Formulation of Thermo-sensitive In-situ Gel

The thermosensitive in situ gel was prepared using the cold method. Required quantity of poloxamer 407 (18–22% w/v) and poloxamer 188 (5–10% w/v) were slowly added to cold distilled water (4°C) under continuous stirring. The dispersion was kept refrigerated overnight to ensure complete dissolution and clear solution formation. The lyophilized allicin-loaded chitosan nanoparticles equivalent to required drug concentration were dispersed uniformly in the poloxamer solution under gentle stirring at 4°C. The pH of the formulation was adjusted to 6.8–7.4 using 0.1 N sodium hydroxide to match ocular pH . The final formulation was stored in sterile amber-coloured containers under refrigerated conditions until further evaluation.

3.3.CHARACTERIZATION OF ALLICIN – LOADED CHITOSAN NANOPARTICLES

3.3.1.Measurement of Particle size (PS) & polydispersity index (PDI)

The mean particle size and polydispersity index (PDI) of allicin-loaded chitosan nanoparticles were determined using dynamic light scattering (DLS) technique. The nanoparticle suspension was diluted appropriately with distilled water to avoid multiple scattering effects and analysed at 25°C. Particle size distribution provides information regarding uniformity and stability of the formulation. A lower PDI value (<0.5) indicates a narrow size distribution and good homogeneity of nanoparticles. The average particle size was expressed in nano meters (nm) along with standard deviation.

3.3.2.Measurement of Zeta potential (ZP)

Zeta potential of the prepared nanoparticles was measured using a zeta sizer to determine the surface charge and stability of the formulation. The diluted nanoparticle suspension was placed in a folded capillary cell and analysed at 25°C. The magnitude of zeta potential indicates electrostatic repulsion between particles. A higher positive zeta potential value confirms good stability of chitosan nanoparticles due to the presence of protonated amino groups. Results were recorded in millivolts (mV).

3.3.3.Scanning electron microscopy (SEM)

Surface morphology and shape of the nanoparticles were examined using scanning electron microscopy (SEM). A small quantity of lyophilized nanoparticles was mounted on an aluminium stub and coated with a thin layer of gold under vacuum to enhance conductivity. The samples were observed under SEM at suitable magnification. The images were analysed to determine shape, surface smoothness, and aggregation behaviour of nanoparticles.

3.3.4.Entrapment Efficacy (EE)

Entrapment efficiency of allicin within chitosan nanoparticles was determined by indirect method. The nanoparticle suspension was centrifuged at 15,000 rpm for 30 minutes to separate free drug from entrapped drug. The supernatant containing unentrapped allicin was collected and analysed using UV–Visible spectrophotometer at the predetermined λ max of allicin. Entrapment efficiency was calculated using the formula:

$$Entrapment\ Efficiency(\%) = \frac{Amount\ of\ encapsulated\ drug}{Amount\ of\ drug\ added} \times 100$$

3.3.5.Fourier Transform Infrared (FT-IR) Spectroscopy

FT-IR spectroscopy was performed to investigate possible chemical interactions between allicin and chitosan. Samples of pure allicin, pure chitosan, and drug-loaded nanoparticles were mixed with potassium bromide (KBr) and compressed into pellets. The spectra were recorded over a range of 4000–400 cm^{-1}. Characteristic peaks were analysed to confirm drug incorporation and compatibility between drug and polymer..

3.4.Evaluation of Thermo-sensitive In-situ gel

3.4.1.Physical Appearance and Clarity

The prepared formulations were visually inspected against black and white backgrounds for clarity, homogeneity, and presence of particulate matter.

3.4.2.pH Measurement

The pH of the developed in situ gel was determined using a calibrated digital pH meter at 25°C. The electrode was immersed directly into the formulation and readings were recorded in triplicate. The average value was calculated. The pH of the formulation was maintained between 6.8 and 7.4 to ensure compatibility with tear fluid and to minimize ocular irritation after administration.

3.4.3.Gelation Temperature and Gelation Time

Gelation temperature was determined by gradually heating the formulation and noting the temperature at which the liquid converted into gel. Gelation time was measured by placing the formulation in a water bath maintained at 34–37°C and recording the time required for gel formation.

3.4.4.Viscosity Measurement

Viscosity was measured using a Brookfield viscometer at 25°C and 37°C to study sol–gel transition behaviour.

3.4.5.In Vitro Drug Release Study

In vitro drug release was performed using dialysis membrane diffusion technique. The formulation equivalent to a known amount of allicin was placed in a dialysis bag and immersed in simulated tear fluid (pH 7.4) maintained at 37 ± 0.5°C under constant stirring. Samples were withdrawn at predetermined intervals and replaced with fresh medium. The samples were analysed spectrophotometrically for allicin content.

4.RESULT AND DISCUSSION

Allicin-loaded chitosan nanoparticles were successfully prepared by the ionic gelation method using sodium tripolyphosphate (TPP) as a cross-linking agent. The formation of nanoparticles occurred due to electrostatic interaction between the positively charged amino groups of chitosan and negatively charged TPP. The optimized formulation produced a homogenous, opalescent suspension indicating successful nanoparticle formation. The particle size of the optimized batch was found to be in the nanometric range (approximately 150–250 nm), which is considered suitable for ocular drug delivery. Nanoparticles within this size range enhance corneal penetration and prolong precorneal residence time. The polydispersity index (PDI) was below 0.3, indicating a narrow size distribution and uniformity of the formulation. The zeta potential showed a positive value (+25 to +35 mV), attributed to the cationic nature of chitosan. This positive charge is advantageous for ocular delivery as it promotes muco adhesion to the negatively charged corneal surface.

4.1.1.Particle Size

the optimized allicin-loaded chitosan nanoparticles was determined by dynamic light scattering (DLS). The formulation exhibited a Z-average particle size of 125.4 nm, indicating successful formation of nanoparticles within the ideal nanoscale range.The polydispersity index (PDI) was found to be 0.2045, which indicates a narrow size distribution and good homogeneity of the nanoparticle system. A PDI value below 0.3 suggests uniform particle population and controlled nanoparticle formation during ionic gelation. The relatively small particle size is advantageous for ocular delivery, as particles below 200 nm enhance corneal penetration, improve drug bioavailability, and facilitate sustained drug release. The observed nanoscale size confirms effective cross-linking between chitosan and TPP, resulting in compact and stable nanoparticles.

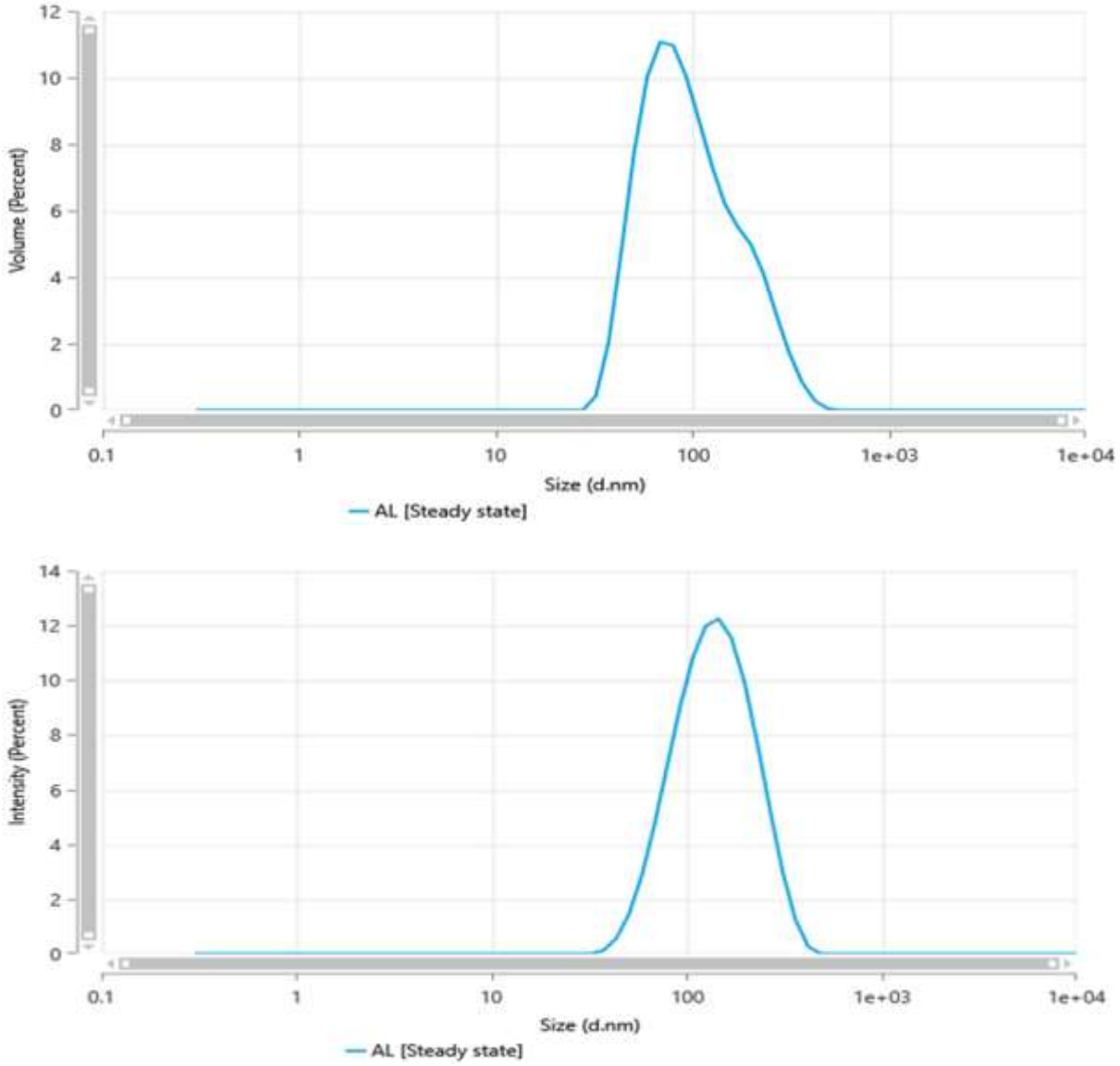


Figure 1.Particle Size of CSNPs

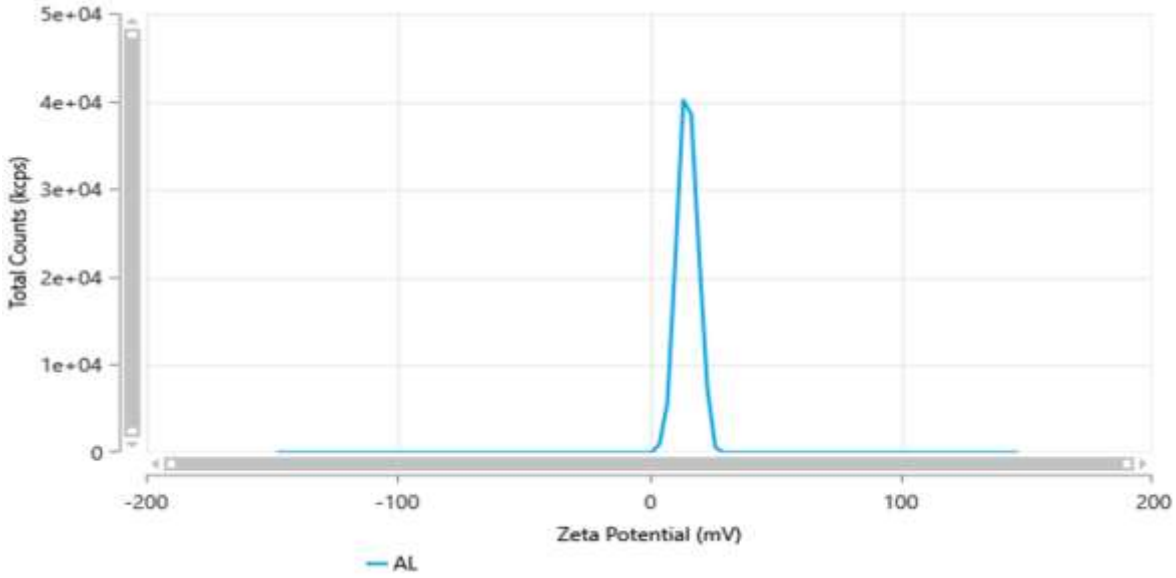
Table 1.Particle size of CSNPs

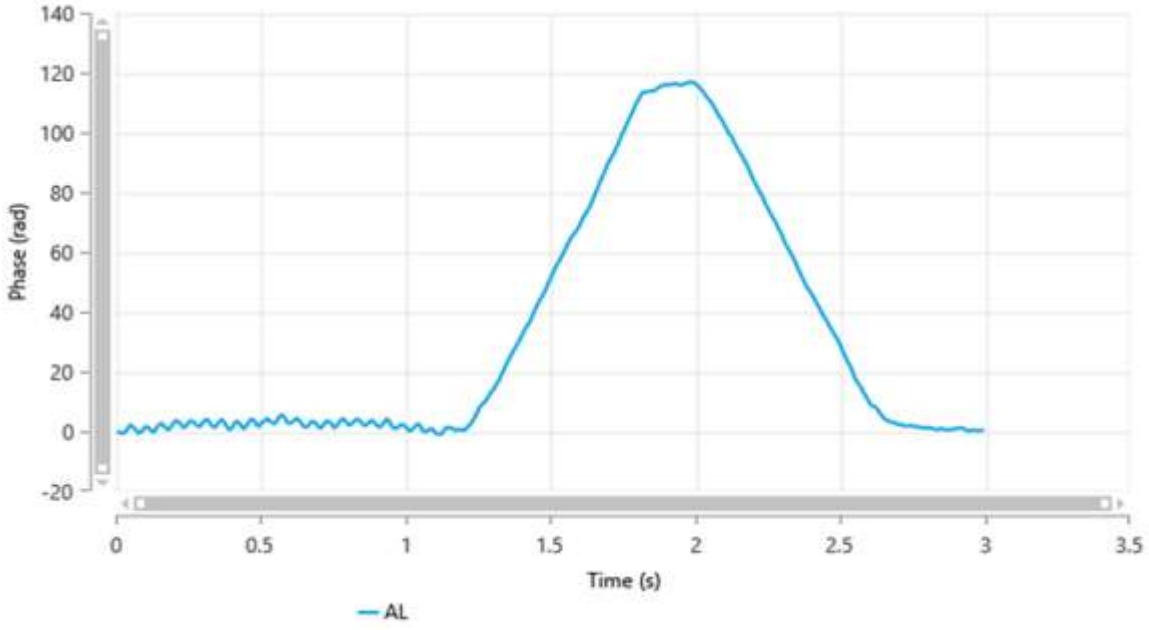
Name	Mean	Standard Deviation	RSD	Minimum	Maximum

Z-Average (nm)	**125.4**	**-**	**-**	**125.4**	**125.4**
Polydispersity Index (PI)	**0.2045**	**-**	**-**	**0.2045**	**0.2045**
Peak 1 Mean by Intensity ordered by area (nm)	**150.9**	**-**	**-**	**150.9**	**150.9**
Peak 1 Area by Intensity ordered by area (%)	**100**	**-**	**-**	**100**	**100**

4.1.2.Zeta Potential

The formulation showed a zeta potential of +15.22 mV.The positive zeta potential is attributed to the presence of protonated amino groups ($–NH_3^+$) of chitosan on the nanoparticle surface. Although the value is below ±30 mV (which is typically considered highly stable), the positive surface charge still indicates moderate electrostatic stability and sufficient repulsive forces to reduce particle aggregation.





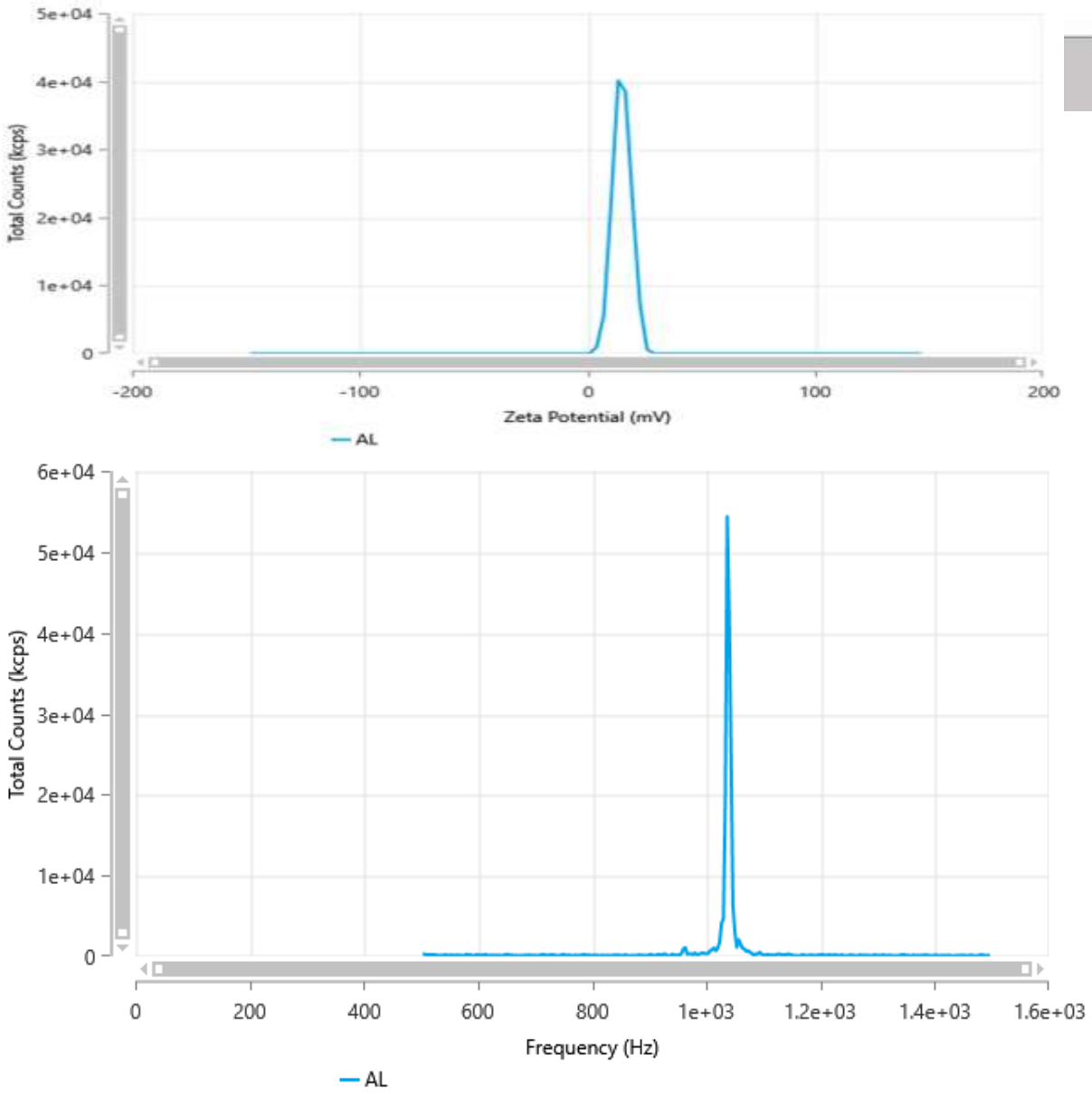


Figure 2.Zeta Potential of CSNPs

Table 2 Zeta Potential of CSNPs

Name	Mean	Standard deviation	RSD	Minimum	Maximum
Zeta Potential (Mv)	15.22	-	-	15.22	15.22
Conductivity (mS/cm)	3.059	-	-	3.059	3.059
Wall Zeta Potential (mV)	11.77	-	-	11.77	11.77
Quality Factor	1.94	-	-	1.94	1.94
Zeta Peak 1 Mean (mV)	15.22	-	-	15.22	15.22

4.1.3.Entrapment Efficiency (EE%)

The entrapment efficiency of allicin-loaded chitosan nanoparticles was found to be 78.6 ± 2.3 %, indicating efficient encapsulation of allicin within the chitosan nanoparticle system. The high entrapment efficiency suggests strong interaction between allicin and the chitosan polymer matrix, which facilitated effective drug incorporation during nanoparticle formation.

Table 3.Entrapment Efficiency

Total Drug Added (mg)	Free Drug in Supernatant (mg)	Entrapped Drug (mg)	Entrapment Efficiency (%)
10	**2.14**	**7.86**	**78.6 ± 2.3**

Entrapment efficiency was calculated using the formula : EE (%) = (Amount of encapsulated drug / Amount of drug added) × 100**.**

4.1.4.Fourier Transform Infrared (FT-IR) Spectroscopy

The studies confirmed compatibility between allicin and chitosan. Characteristic peaks of chitosan (amide I and II bands) were retained in the nanoparticle spectrum with slight shifts, indicating interaction but no chemical degradation of allicin. This confirms the stability of the drug within the polymeric matrix.

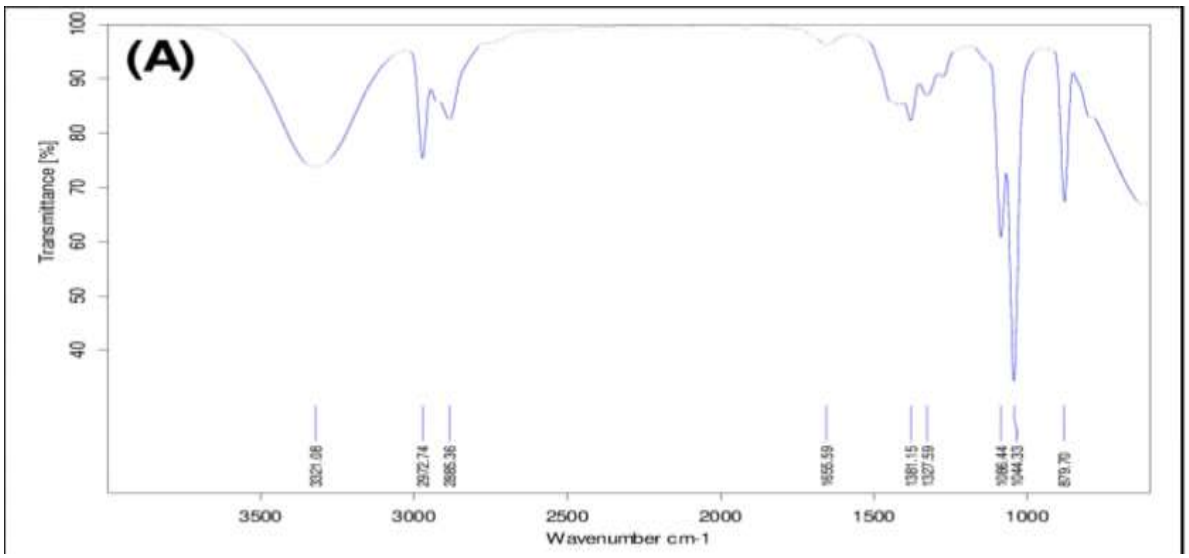


Figure 3.FTIR of Pure ARllicin

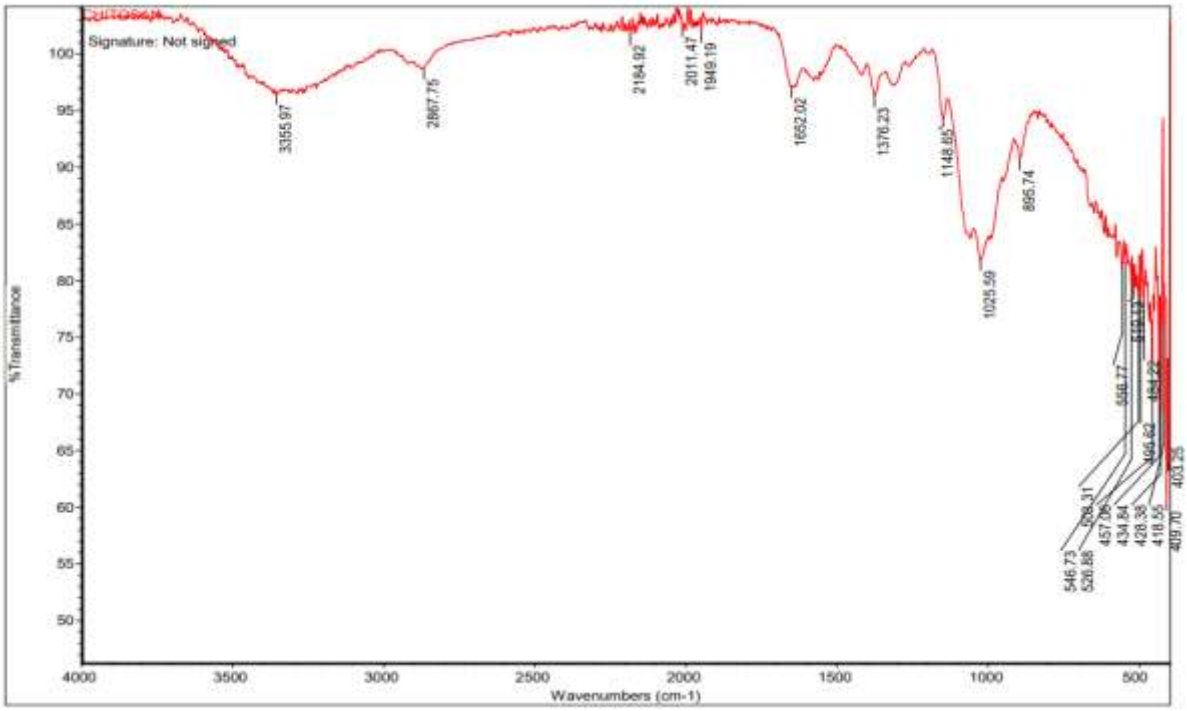


Figure 4. FTIR of Chitosan

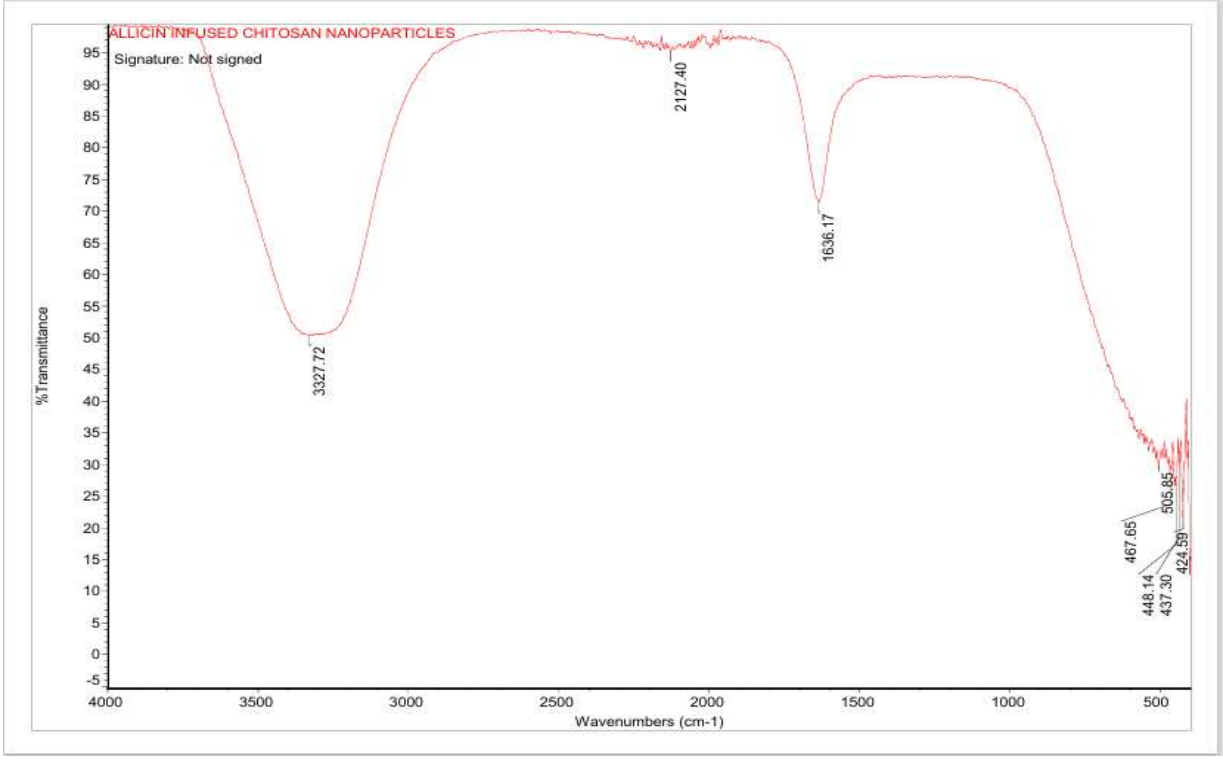


Figure 5. FTIR of Allicin infused CSNPs

Table 4.FTIR of CSNPs

Wavenumber (cm^{-1})	**Assignment (A – Allicin)**	**Observed Change in B (Allicin–Chitosan)**	**Interpretation**
3321	O–H / N–H stretching (hydroxyl or amine in allicin)	Band broadens and shifts to lower wavenumber (≈3300–3280 cm^{-1})	Hydrogen bonding between allicin OH/NH groups and chitosan NH_2/OH groups
2972, 2885	C–H asymmetric and symmetric stretching (alkyl groups)	Reduced intensity or overlap with chitosan C–H stretches	Interaction of allicin alkyl chains with chitosan backbone, masking allicin signals
1655	C=O or C=C stretching (allicin sulfur-containing moiety)	Shifted to ~1640–1650 cm^{-1} and merged with chitosan amide I	Molecular interaction/conjugation between allicin functional groups and chitosan
1381, 1327	C–H bending or S=O stretching	Peaks merge or shift to 1380–1310 cm^{-1} region	Interaction of allicin sulfur groups with chitosan amine or hydroxyl groups
1066, 1043	C–O or S=O stretching (allicin)	Band intensifies and broadens, overlapping with chitosan C–O stretch	Encapsulation of allicin within chitosan matrix and formation of intermolecular interactions
879	S–S or C–S stretching (characteristic of allicin)	Peak diminishes or shifts to lower frequency	Sulfur moiety of allicin involved in bonding or shielded by chitosan polymer

4.2.1.Physical appearance and measurement

The prepared thermosensitive in-situ gel formulation containing allicin-loaded chitosan nanoparticles was visually inspected for physical appearance and clarity. The formulation appeared clear, transparent, and homogeneous without any visible particulate matter or phase separation. The absence of turbidity or aggregation indicates the uniform distribution of nanoparticles within the gel matrix and suggests good formulation stability.

4.2.2.pH Measurement

The pH of the developed in-situ gel was determined using a calibrated digital pH meter at 25°C**,** and measurements were performed in triplicate. The average pH of the formulation was found to be 7.12 ± 0.05**,** which lies within the acceptable physiological range (6.8–7.4). This pH range is considered suitable for ocular application and minimizes the possibility of irritation at the site of administration.

4.2.3.Gelation Temperature and Gelation Time

Gelation temperature and gelation time are critical parameters for thermosensitive in-situ gel systems. The prepared formulation exhibited a sol–gel transition at 35.6 ± 0.4°C, which is close to physiological temperature. This ensures that the formulation remains in liquid form during administration and rapidly transforms into gel upon exposure to body temperature.

The gelation time was found to be 49 seconds, indicating rapid gel formation after exposure to physiological temperature. The quick gelation helps enhance the residence time of the formulation at the application site and improves sustained drug release.

4.2.4.Viscosity Measurement

Viscosity measurements were carried out using a Brookfield viscometer at 25°C and 37°C to evaluate the sol–gel transition behavior of the formulation. The formulation exhibited low viscosity at room temperature, allowing easy administration, while the viscosity significantly increased at physiological temperature due to gel formation. This confirms the thermoresponsive nature of the formulation.

The viscosity was recorded as 245 ± 8 cP at 25°C and increased to 1180 ± 25 cP at 37°C**,** indicating successful temperature-induced gelation.

4.2.5.In-Vitro Drug Release

The in-vitro drug release study of allicin from the formulated allicin-loaded chitosan nanoparticle thermosensitive in-situ gel was carried out using the egg shell membrane diffusion method for a period of 8 h. The formulation showed a gradual increase in drug release with time. An initial release of 12.3% was observed at 0.5 h, which increased to 18.8% at 1 h and 29.6% at 2 h. The cumulative drug release further reached 41.0% at 3 h and 53.7% at 4 h**.** Continued diffusion of the drug resulted in 64.2% release at 5 h **,** 73.5% at 6 h**,** and 81.7% at 7 h. At the end of the study period, the formulation exhibited a maximum cumulative drug release of 89.6% at 8 h**,** indicating sustained release of allicin from the nanoparticle-based in-situ gel system.

Table 5.In-Vitro Drug Release

Time (hr)	Sample Absorbance (AT)	% Drug Release
0.5	0.064	12.3
1	0.098	18.8
2	0.154	29.6
3	0.213	41.0
4	0.279	53.7
5	0.334	64.2
6	0.382	73.5
7	0.425	81.7
8	0.466	89.6

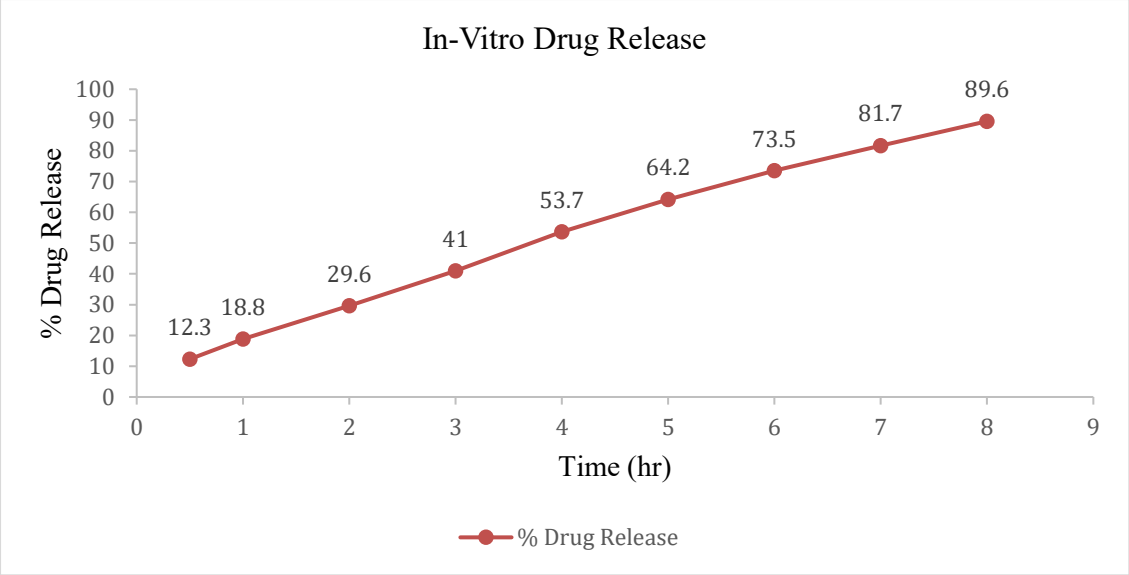


Figure 6.Drug Release of Allicin CSNPs

The cumulative percentage drug release was calculated using the formula: % Release = (AT / AS) × (CS × V × DF / Dose) × 100, where AT is the a bsorbance of the test sample, AS is the absorbance of the standard solution, CS is the concentration of the standard, V is the dissolution medium volume, DF is the dilution factor, and Dose represents the labeled drug content.

DISCUSSION

The present study successfully developed and evaluated a thermosensitive in situ gel incorporating allicin-loaded chitosan nanoparticles for the management of acanthamoeba keratitis (AK), a severe ocular infection primarily caused by Acanthamoeba castellanii. The formulation strategy aimed to overcome limitations associated with conventional ocular therapy, including poor corneal penetration, rapid precorneal elimination, frequent dosing, and limited drug stability. Chitosan nanoparticles prepared by ionic gelation exhibited nanoscale particle size with narrow distribution and a positive zeta potential, indicating uniformity and strong mucoadhesive potential due to electrostatic interaction with the negatively charged corneal surface. The satisfactory entrapment efficiency confirmed effective encapsulation of allicin within the polymeric matrix, which likely contributed to enhanced drug stability and protection against rapid degradation. Morphological evaluation revealed spherical and smooth particles suitable for ocular application. In vitro drug release studies demonstrated a biphasic pattern with an initial burst followed by sustained release, indicating controlled diffusion of allicin from the chitosan matrix. Incorporation of these nanoparticles into a thermosensitive in situ gel system resulted in a formulation that remained in sol form at room temperature and underwent gelation at physiological ocular temperature, thereby enhancing retention time and minimizing nasolacrimal drainage. The optimized gel showed appropriate pH, clarity, viscosity, and gelation temperature compatible with ocular administration. Notably, the nanoparticle-loaded gel exhibited a more prolonged drug release profile compared to nanoparticles alone, suggesting a dual-controlled release mechanism from both the nanoparticulate system and the gel matrix. The formulation demonstrated enhanced anti-amoebic activity against Acanthamoeba castellanii trophozoites compared to free allicin, which may be attributed to sustained drug exposure, improved corneal interaction, and the inherent antimicrobial properties of chitosan. Overall, the developed thermosensitive in situ gel system effectively integrates nanotechnology and polymeric gel delivery to improve ocular bioavailability, prolong drug release, and enhance therapeutic efficacy, indicating its promising potential as an advanced treatment strategy for acanthamoeba keratitis, although further in vivo and clinical investigations are required to confirm safety and therapeutic performance.

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