

PIPERINE-LOADED CHITOSAN NANOPARTICLES: PREPARATION AND CHARACTERIZATION

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Abstract. Plasma cholesterol level plays an important role in atherosclerosis and cardiovascular diseases. Treatment of cardiovascular diseases has become one of the major focuses of scientific and technological development in recent years. Piperine (PIP), an alkaloid form of black pepper is known to reduce cholesterol uptake. Nanoparticles offer numerous advantages as compared to microparticles. Chitosan is a non-toxic biodegradable polycationic polymer that has been extensively investigated. Nanoparticles based on chitosan being biodegradable, biocompatible, less toxic and easy to prepare, are an effective and potential tool for drug delivery. In this paper, piperine-loaded chitosan nanoparticles (CTS-PIP NPs) were prepared by ionic gelation method. Molecular interactions among the components were confirmed by Fourier-transform infrared spectroscopy (FTIR) spectroscopy. The morphology of the prepared NPs was characterized by transmission electron microscopy image (TEM). The TEM analysis indicated that PIP-CTS NPs were spherical-shaped and well-separated with diameter of < 100 nm. CTS-PIP NPs displayed positive ζ -potential (ZP) of about 31.6 mV. Dynamic light scattering (DLS) and particle size (PS) distribution analysis indicated the mean particle size of CTS-PIP NPs was 245.9 nm, polydispersity index of 31 %. Results of the stability study revealed that insignificant changes in zeta potential and polydispersity of CTS-PIP NPs after three months.

Keywords: piperine, chitosan, nanoparticles.

Classification numbers: 2.4.3, 2.7.1, 1.2.4.

1. INTRODUCTION

Chitosan (CTS) is a linear copolymer of β -(1 \rightarrow 4)-linked 2-acetamido-2-deoxy- β -D-glucopyranose and 2-amino-2-deoxy- β -D-glucopyranose. CTS possesses unique properties such as biocompatibility, biodegradability, hydrophilicity, nontoxicity and high bioavailability [1]. It can be processed into films, gels, nanoparticles (NPs), microparticles and beads [1, 2]. CTS is dissolved in an acidic medium, its amino groups in the polymeric chains are protonated and become cationic, which allows its strong interaction with different kinds of molecules. The results of *in vitro* and *in vivo* studies demonstrated that chitosan is effective in lowering blood cholesterol. The hypocholesterolaemic activity of chitosan was proved to be better when a degree of deacetylation was high, which might be due to the electrostatic force between chitosan

and anion substances, such as fatty acid and bile acid [2, 3]. In addition, the biodegradable CTS is broken down in the human body to safe compounds (amino sugars), which are easily absorbed.

CTS and its derivatives are broadly investigated in numerous pharmaceutical and medical applications, especially for drug carriers. Various methods for the preparation of chitosan nanoparticles (CTS NPs) include: ionic cross-linking, covalent cross-linking, reverse micelle method, precipitation and emulsion-droplet coalescence method [4]. Pharmaceutical carriers such as polymers, micelles, liposomes and nanoparticles have received increased attention. These systems reveal numerous advantages principally in enhanced efficacy and safety of the drugs. These systems can incorporate both hydrophobic and hydrophilic active compounds, which depends on carrier nature. CTS NPs are found to have a plethora of applications in drug delivery diagnosis and other biological applications.

Natural products are considered as important sources of new drugs. The alkaloids have diverse biological and pharmacological activities. Piperine (PIP) is an alkaloid found in several species of piper, mainly *Piper nigrum* Linn. and *P. longum*. PIP has many pharmacological properties, such as antidiabetic, antidiarrheal, antioxidant, antibacterial, and antiparasitic activity [5]. It also reduces cholesterol uptake and enhances translocation of cholesterol transporter proteins, as reported by Christian Rafael Quizia *et al.* [5]. PIP-loaded NPs showed a more significant inhibitory effect on seizure-related behavioral signs compared to the free PIP [6]. The anticonvulsant property of PIP -loaded NPs is partly mediated through its inhibitory effects on neuronal loss and astrocytes activation in fully kindled animals [7]. Hydrophobic drug PIP could also be successively loaded in hydrophilic CTS NPs with high entrapment and zeta potential sufficient for stabilization. PIP is a known irritant of nasal mucosa due to its pungency. One of the advantages of using CTS NPs as a drug delivery system is the ability of CTS NPs to encapsulate PIP and slowly release it to minimize the concentration of the drug in direct contact with nasal mucosa to prevent nasal irritation. The nanoparticles acted as brain-targeted therapy in Alzheimer's disease [8].

Diana Anissian has prepared PIP-loaded chitosan-sodium tripolyphosphate (TPP) nanoparticles and the effect of PIP NPs on seizures behavior and astrocytes activation was assessed in pentylenetetrazol (PTZ)-induced kindling model [7]. The author focused mainly on bioactivities of the products. Hypertension and hypercholesterolemia are the main factors of morbidity and mortality in today's society since they are two key risk factors for the emergence and development of cardiovascular diseases. Both CTS and PIP could reduce cholesterol uptake and enhance translocation of cholesterol transporter proteins [2, 3, 5]. The preparation of NPs intended to be used as functional food to support for the treatment of hypercholesterolemia rarely been reported before. Our preliminary optimal conditions for preparation of CTS-TPP nanoparticles have been investigated [9]. This is the first step toward this aim. In this paper, the preparation and characterization (FTIR, TEM, size distribution, zeta potential and storage stability) of PIP-loaded chitosan nanoparticles. To the best of our knowledge, such research has never been reported in Viet Nam before.

2. MATERIALS AND METHODS

2.1. Materials

Chitosan with medium molecular weight ($M_w \cong 100$ kDa) and degree of deacetylation of 90 % were prepared in our lab as described before [10]. PIP and sodium tripolyphosphate were

purchased from Sigma–Aldrich Chemical Co. Ltd. Tween 80, glacial acetic acid, ethanol AR, and all other reagents were of analytical grade.

2.2. Methods

CTS NPs were prepared by ionic gelation method. PIP was incorporated into NPs by encapsulation method. Based on our preliminary experimental results [9] and Quan Gan *et al.* [11], CTS-PIP NPs were prepared as followed: chitosan was dissolved in 1 % acetic acid to reach a final concentration of 2 mg/ml. Tween 80 (1 %) was added into 100 ml chitosan solution (2 mg/ml) and sonicated for 15 min. pH of the solution was then adjusted to 5 using NaOH solution (2N). PIP in acetic acid in different concentrations (0 ÷ 3000 µg/ml) was added to CTS solution and sonicated for 5 min. Sodium TPP in deionized water (CTS:TPP ratio of 5:1 w/w) was added dropwise with a syringe under stirring. The prepared dispersions were allowed to stabilize by magnetic stirring for 60 min.

CTS- PIP NPs were collected by centrifugation (15,000 rpm for 30 min). The precipitate was re-dispersed in 5 ml deionized water by sonication for 10 min. Then lyophilization was performed in the freeze dryer ALPHA 1-4 LD (Germany) for further physicochemical investigation. The supernatant was collected, filtered through a Millipore membrane filter (0.45 µm), and used to determine untrapped PIP using a UV spectrophotometer at 342 nm. The percentage of encapsulation efficiency (EE) of the prepared NPs was calculated by the following equation:

$$EE (\%) = \frac{\text{Total amount of piperine} - \text{Free Piperine}}{\text{Total amount of Piperine}} \times 100.$$

TEM images, IR spectra, particle size (PS), zeta potential (ZP) and polydispersity index (PI) of the aforementioned prepared PIP-loaded CTS-NPs were investigated.

The physicochemical stability: CTS-PIP NPs were packed into screw-capped glass vials and stored at 30 ± 1 °C, away from direct sunlight. The changes in the particle size, zeta potential and polydispersity were observed over 1 week, 1 month and 3 months.

The morphological characteristics of NPs were observed using a transmission electron microscopy (TEM) JEOL - JEM 1010, National Institute of Hygiene and Epidemiology. A drop of nanosuspension was placed on a paraffin sheet and carbon-coated grid was placed on sample and left for 1 min to allow the NPs to adhere on the carbon substrate. The remaining suspension was removed by adsorbing the drop with the corner of a piece of filter paper. The samples were air dried before microscopic investigation.

Physicochemical characterization of CTS-PIP NPs: The PS, PDI, and ZP were determined by dynamic light scattering (DLS) technique using Litesizer 500 (Anton Paar GmbH), Institute of Chemistry. All samples were measured in triplicates and results were represented as mean value \pm SD.

FTIR spectra of pure CTS, piperine, and CTS-PIP NPs were recorded using a Nicolet Nexus 760 FT-IR spectrometer in the range of 500 - 4000 cm^{-1} , Institute of Chemistry - Vietnam Academy of Science and Technology.

3. RESULTS AND DISCUSSION

3.1. Physicochemical elucidation of PIP-loaded CTS-NPs.

3.1.1. Encapsulation efficiency, particle size, zeta potential, and polydispersity

Chitosan nanoparticles were formed by ionic gelation technique between positively charged CTS and negatively charged TPP. PIP was incorporated into NPs by encapsulation method. The particle size of NPs is one of the most significant determinants in mucosal and epithelial tissue uptake and intracellular trafficking [12]. Surface charge is another important determinant in the stability, mucoadhesiveness, and permeation enhancing effect and the ability of NPs to escape from the endolysosomes [13]. Our prepared CTS NPs suspension was light yellow, opaque color. All the suspensions did not appear macroscopically considerable aggregates.

PIP is an alkaloid, when added to CTS solution it interacted with TPP during NPs fabrication (encapsulation) or formed a hydrogen bond with CTS particles on the surface. Mean particle size, polydispersity index (PDI), ζ -potential, encapsulation efficiency (EE) of NPs with different initial PIP concentrations were shown in Table 1. The mean particle size of blank CTS NPs was found to be 169 nm. After PIP encapsulation, the particle size increased from 205 nm to 324 nm when PIP concentration increased from 250 to 3000 μg . At low PIP concentration (250 $\mu\text{g}/\text{ml}$), the amount of positively charged PIP will be much less compared with that of CTS leading to a very low possibility to interact with TPP. Increasing the amount of PIP to 2000 $\mu\text{g}/\text{ml}$ led to higher chance of interaction and consequently, the amount entrapped increased till reaching the saturation solubility, leading to increase in particles size diameter and encapsulation efficiency as indicated in the Table 1. However, when the PIP concentration was higher than 2000 $\mu\text{g}/\text{ml}$, the PS increased but EE decreased. This may due to the concentration saturation solubility of PIP in the nanosuspension solution was 2000 $\mu\text{g}/\text{ml}$.

Table 1. Mean particles size, polydispersity index, zeta potential and encapsulation efficiency (EE).

Sample	PIP ($\mu\text{g}/\text{ml}$)	Particle size (nm)	ζ - potential	PDI	EE (%)
CTS-PIP NPs 0	0	169 \pm 3.66	+25.2	0.22	-
CTS-PIP NPs 1	250	205 \pm 2.55	+32.3	0.23	23
CTS-PIP NPs 2	500	218.5 \pm 3.04	+33.1	0.25	45
CTS-PIP NPs 3	1000	232.1 \pm 1.23	+31.8	0.28	62
CTS-PIP NPs 4	2000	245.9 \pm 2.14	+31.6	0.31	85
CTS-PIP NPs 5	3000	324 \pm 1.05	+31.3	0.48	80

PDI is a measure of the homogeneity of the particles. The PDI of CTS-PIP NPs was between 0.23 and 0.48, which indicated that a homogeneous dispersion of CTS-PIP NPs with narrow dispersity was obtained. Zeta potential is a measure of the particle surface charges. Particle charge is stability-determining parameter in aqueous nanosuspensions. Results indicated that PIP-CTS NPs possessed a positive zeta potential of about 31 mV that was considered stable. When added to CTS solution at pH of 5.0, PIP would adopt a positive charge and thereby interacted with TPP during NPs fabrication (encapsulation) or formed hydrogen bond with CTS particles on the surface by adsorption that made the PIP-CTS NPs more stable and had zeta potential higher than that of CTS NPs. Non-significant variations in zeta potential could be observed. The positive zeta potential value was due to the cationic nature of chitosan. Mean particle size, polydispersity index, zeta potential and encapsulation efficiency (EE) of PIP-CTS NPs were shown in Table 1. Figure 1 and Figure 2 showed the corresponding size distribution

curve and zeta potential curve of PIP-CTS NPs suspension at the PIP encapsulation concentration of 2000 μg (CTS-PIP NPs 4), respectively.

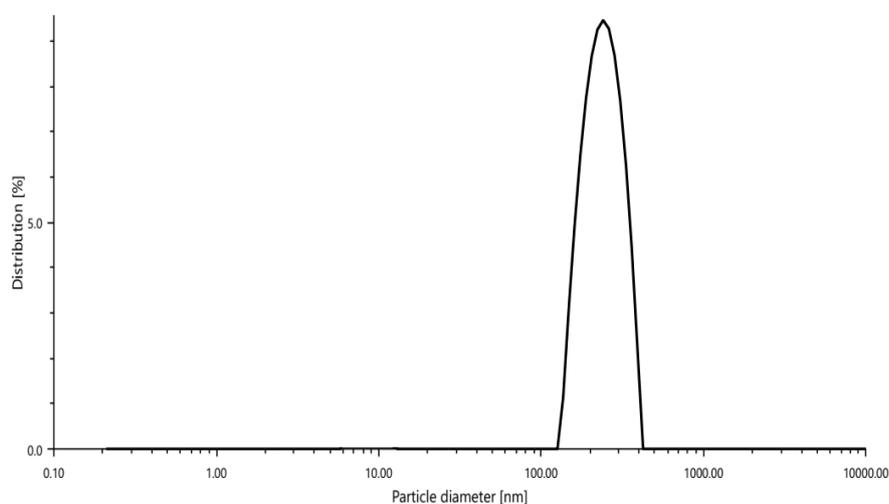


Figure 1. Size distribution of CTS-PIP NPs 4.

Zeta potential distribution

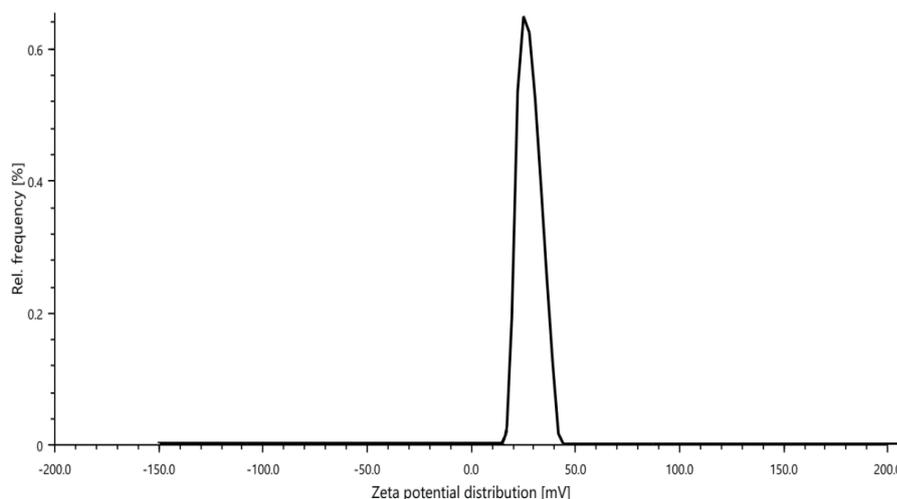


Figure 2. Zeta potential of CTS-PIP NPs 4.

3.1.2. Morphological characterization

TEM imaging is widely used to investigate NPs morphology, as well as their size. Figure 3 showed the morphological characteristic of PIP-CTS NPs 4 by TEM image. The TEM analysis indicated that PIP-CTS NPs were almost spherical-shaped in their morphology and well separated and discrete from each other, indicating promising stability of nanoparticles. The TEM images exhibited a smaller diameter than that obtained from the DLS measurements (< 100 nm). This may be due to the shrinking and separation of the NPs during the drying process, as indicated by Bing Hu [13] and Musaed Alkholief [14].

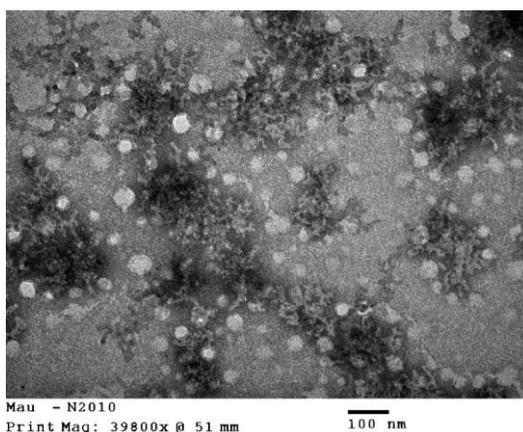


Figure 3. TEM image of CTS-PIP NPs 4.

3.1.3. FTIR analyses

FTIR study was performed to evaluate the chemical interaction of components used in the elaboration of the nanoparticles. The interactions were detected by the variation of peak shape, position and intensity. The FTIR spectra of PIP, CTS and PIP-CTS NPs were presented in Figure 4, and FTIR analyses were shown in Table 2.

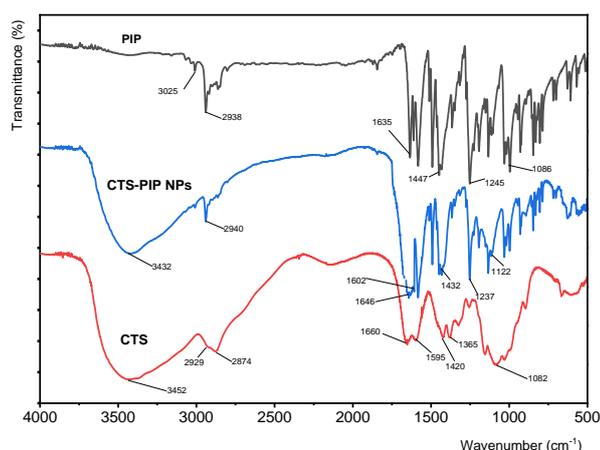


Figure 4. Infrared spectra of chitosan, piperine and PIP-CTS NPs.

As indicated in Figure 4, upon the NPs formation, the shoulder peak at 1660 cm^{-1} decreased significantly and a new peak appeared at 1646 cm^{-1} . Moreover, the amide II peak at 1595 cm^{-1} in chitosan became weak and shifted to 1602 cm^{-1} in PIP-CTS NPs, confirming that amine groups of chitosan were involved in electrostatic interactions with phosphate groups of TPP. Furthermore, there are some strong electronegative atoms like N and O in the piperine molecule. When piperine was incorporated into PIP-CTS NPs, the hydroxyl group peak of chitosan changed from 3452 cm^{-1} to 3432 cm^{-1} , suggesting that there were some hydrogen bonds existed between piperine and chitosan. On the other hand, PIP was also incorporated into PIP-CTS NPs during nanoparticle formation, with a great possibility of interacting with anionic sodium triphosphate, as indicated by Elnaggaret *et al.* [8]. These observations also indicated that no

chemical interaction existed among these groups and the compounds used in the nanoparticles' generation.

Table 2. Absorption bands of chitosan, piperine and PIP-CTS NPs.

Chitosan		Piperine		CTS-PIP NPs	
Wave-number (cm ⁻¹)	Associated vibrations of bonds	Wave-number (cm ⁻¹)	Associated vibrations of bonds	Wavenumber (cm ⁻¹)	Associated vibrations of bonds
3542	O-Hand N-H stretching	3025	=C-H stretching	3432	O-Hand N-H stretching
2874	C-H stretching	2938	alkane C-H stretching	2940	C-H stretching
1660, 1595	N-H bending from amine and amide II	1635, 1579	C=O or conjugated C=C stretching	1646, 1602	N-H bending from amine and amide II
1420	-CH ₂ bending	1447	C-H bending	1432	-CH ₂ bending
1365	anti-symmetric stretching of C-O-C and C-H stretching	1245	C-O stretching	1237	C-O stretching
1082	presence of amine groups in CTS	1084	C-N	1086	C-N

3.2. Storage stability

Results of the stability study shown in Table 3 indicated that only a small increase in the size PIP-CTS NPs was observed (from 245 to 269 nm). No significant changes in zeta potential and polydispersity of NPs under the above storage conditions. Lyophilization might facilitate long shelf-life stability of the NPs as indicated by Elnaggar *et al.* [8].

Table 3. Periodic evaluation of PS, ζ - potential, PDI of PIP-CTS NPs during the storage.

Parameters	Time points			
	Initial	1 week	1 month	3 months
Particle size (nm)	245.9 ± 2.14	246 ± 1.53	250 ± 2.12	269 ± 1.12
Zeta potential (mV)	+ 31.6	+ 31.2	+ 30.6	+ 30.2
Polydispersity	0.31	0.30	0.29	0.28

4. CONCLUSIONS

In conclusion, spherical piperine-loaded chitosan nanoparticles with the average particle size smaller than 100 nm have been successfully prepared. PIP-CTS NPs displayed positive ζ -

potential (+ 31.6) with a high degree of homogeneity (PDI 0.31) and EE of 85 %. Electrostatic interactions and hydrogen bonds were a driving force for the formation of PIP-CTS NPs as confirmed by IR spectra. However, further studies will be needed to fully evaluate the ability of the NPs in preventing hypercholesterolaemia.

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Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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