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Characterization of Chitosan Nanoparticles of Sea Urchin (*Tripneustes Gratilla*) Using Beads-Milling Method

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ABSTRACT

Although chitosan from the waste of the sea urchin *Tripneustes gratilla* has the potential to be developed into a nanoparticle product through a variety of evolving methods, it is not yet known which method is most effective for producing high-quality nanoparticles with optimal physicochemical properties and how these methods affect their characteristics and potential applications. This study aimed to characterize chitosan sea urchin nanoparticles (NPs CsSU) produced using the bead-milling method with Tween-80 surfactant at different concentrations. NPs CsSU and TPP were mixed using a dropwise technique to prevent reagglomeration in the suspension, followed by the addition of Tween-80 surfactant at concentrations of 0, 30, 60, and 90% (v/v) to obtain NPs CsSU. The analysis results showed that NPs CsSU had moisture content ranging from 12.73 to 18.37% and lightness (L) values between 60.26 and 88.74. The average particle size (d50) ranged from 75 to 528nm, with the lowest polydispersity index (PI = 0.72) observed at 60% (v/v) Tween-80. The FTIR spectra indicated strong peaks in the range of 3480– 3400cm⁻¹, corresponding to hydrogen-bonded O–H vibrations. SEM analysis revealed more uniform morphology and smaller particle sizes, averaging below 100nm at 60% (v/v) Tween-80. The zeta potential values were 1.94, 3.63, 0.58, and 0.42mV, each showing only one peak. Meanwhile, the XRD spectrum showed a diffraction peak at 2θ = 19.05°, indicating lower crystallinity with an amorphous structure. As a conclusion that characterization of chitosan ion interactions with tween-80 showed an influence in the formation of chitosan sea urchin nanoparticle (NPs CsSU) polymers due to the use of surfactant media in chitosan suspensions using a beads-milling method.

INTRODUCTION

Chitin is a natural polysaccharide found in the shells of crustaceans and in fungal cell walls. In seafood processing, about 60–70% of the raw material becomes waste, much of which is inedible **(Rai *et al.*, 2025; Vega-Baudrit *et al.*, 2025)**. This waste contains 20–40% chitin, making it a valuable but underused resource **(Rai *et al.*, 2025)**. When chitin undergoes deacetylation, it produces chitosan, an abundant biopolymer with many uses in food, agriculture, and pharmaceuticals **(Mersmann *et al.*, 2025)**.

Chitosan is especially attractive because it is biocompatible, biodegradable, and gel-forming. These properties make it useful in biomedical and pharmaceutical fields, particularly in drug delivery systems **(Blebea *et al.*, 2025)**. Conventional drug delivery methods often face challenges like poor solubility, limited absorption, and low bioavailability. Nanoparticles can help solve these problems **(Ragavan *et al.*, 2024)**. Chitosan has received much attention as a nanocarrier because it is non-toxic, degradable, and capable of forming nanoparticles with improved delivery properties **(Biswas *et al.*, 2025)**.

The chemical structure of chitosan includes hydroxyl and amino groups, which allow further modification and expansion of its applications **(Biswas *et al.*, 2025)**. It also has antibacterial properties, influenced by pH, concentration, molecular weight, and degree of deacetylation. These qualities make chitosan useful in antimicrobial applications and eco-friendly packaging **(Chicea & Nicolae-Maranciuc, 2024)**. Structural changes, such as turning it into nanoparticles, further increase its therapeutic effectiveness and bioavailability, broadening its role in nanomedicine **(Ragavan *et al.*, 2024)**.

Chitin is a linear polymer made of poly-β-N-acetyl-D-glucosamine and is the second most abundant natural biopolymer after cellulose **(El-araby *et al.*, 2024)**. Chitosan, derived through deacetylation, is composed of glucosamine and N-acetylglucosamine units linked by glycosidic bonds **(Gonciarz *et al.*, 2025)**. Its antibacterial and antifungal activities make it valuable in developing biomaterials to fight antibiotic resistance **(Dhlamini *et al.*, 2024)**.

Polysaccharides like chitosan and cyclodextrin are widely studied in biomedical research because of their potential in treating cancer, cardiovascular disease, and other health issues **(Bahavarnia *et al.*, 2024)**. Chitosan is further classified into high molecular weight and low molecular weight types. Low molecular weight chitosan is more soluble and biologically active, making it especially effective for drug delivery **(Benalaya *et al.*, 2024; Akdaşçi *et al.*, 2025)**.

Chitosan’s polycationic nature enables it to interact with negatively charged cell components, improving cellular uptake and drug delivery **(Das *et al.*, 2024)**. This is also useful for antimicrobial purposes, as positively charged chitosan binds to bacterial cell membranes, disrupting them and causing inactivation **(Molina-Pinna & Román, 2025)**. Its mucoadhesive properties allow prolonged contact with mucosal tissues, improving absorption and localized treatment, particularly in dentistry **(Roma *et al.*, 2024)**. In addition, chitosan can bind metal ions and form hydrogels, supporting its use in dental restoration and periodontal therapy **(Paczkowska-Walendowska *et al.*, 2025)**.

Improving chitosan properties through nanotechnology is a promising strategy. Methods such as bead-milling can produce uniformly sized nanoparticles that enhance drug encapsulation and allow controlled release **(Ragavan *et al.*, 2024)**. Chitosan nanoparticles can reach encapsulation efficiencies of up to 90%, which is highly beneficial for drug delivery **(Ştefanache *et al.*, 2025)**. With additional surface modifications, these nanoparticles can also enable targeted delivery, making them effective platforms for advanced therapies **(Krasowska *et al.*, 2024)**.

By increasing surface area and dissolution rates, chitosan nanoparticles improve drug solubility and bioavailability, addressing major limitations of conventional drug delivery **(Ragavan *et al.*, 2024)**. Other nanoparticles, such as solid lipid, polymeric, and gold nanoparticles, have also been explored, each with unique advantages and drawbacks. However, chitosan's natural origin, safety, and versatility make it especially promising for future applications **(Krasowska *et al.*, 2024)**. This research is particularly important because it has not been previously reported, making this product novel. Therefore, the aim of this study was to characterize chitosan sea urchin nanoparticle (NPs CsSU) using the bead-milling method.

MATERIALS AND METHODS

1. Materials

The raw materials of waste coconut crabs were received from waste restaurant of Ternate City, North Maluku, Indonesia. They were ball mill into powder and followed by the deacetylation process to obtain chitosan sea urchin (named Cs SU) with deacetylation value of 89% and molecular weight 368 kDa. The tripolyphosphate as biopolymer matrix was purchased from Sigma-Aldrich, St. Louis, MO, USA. The Tween-80 as a dispersing agent was purchased from PT. Brataco Chem, Indonesia, and glacial acetic acid were received from PT Merck Indonesia.

2. Preparation of chitosan sea urchin nanoparticle (NPs CsSU)

Chitosan sea urchin (Cs SU) powder 0.25 g was dissolved into 100 mL acetic acid 1 (% v/v). 0.003 TPP was dissolved (% w/v) in distilled water and mixed using a magnetic stirrer with a rotational speed of 600 rpm for 30 minutes. Furthermore, TPP were dropwised into dissolved chitosan sea urchin to prevent past precipitation or reagglomeration of the suspension. Finally, Tween-80 was added to suspend with concentrations (0, 30, 60, and 90 (% v/v) and continuously sonicated at λ 60 kHz for 30 minutes **(Zhang *et al.*, 2005)** before beads-milling process for 2 hours. The obtained suspension named chitosan sea urchin nanoparticles (NPs CsSU) was then filtered using 400 mesh to separate zirconia beads.

3. Characterization of NPs CsSU

Before the preparation of films, the as-prepared chitosan sea urchin nanoparticles (NPs CsSU) powder were subjected to analysis including their morphology using scanning electron microscopy (SEM), size and size distribution using particle size analysis (PSA), present of Tween-80 as dispersing agent using Fourier-transform infrared spectroscopy (FT-IR) and stability of dispersion using zeta potential, and amorphous phase using X-Ray Diffraction (X-RD).

A. *Water content and color of NPs CsSU*

Determination of NPs CsSU water content (AOAC 930.15) was according to standard methods of **AOAC (2004)**. The color values (L*, a*, b*) of the NPs CsSU were measured with CR 310 Minolta Chroma Meters (Minolta Camera Co., Ltd). The NPs CsSU sample was placed on a white standard plate (calibration plate) and the Hunter Lab color scale was used to measure color. Each sample was measured at four different reading positions.

B. *Particle size analysis of NPs CsSU*

The NPs CsSU suspension from the centrifugation process was suspended in 20 mL of distilled water. To obtain an observable suspension, 1 (% v/v) of this suspension was dissolved in 70ml of distilled water. This suspension was then included in the size distribution analysis using particle size analysis (PSA, Beckman Coulter LS 13320). This instrument provides analytical applications with the Delsa™ Nano Series instrument that uses dynamic light scattering (DLS) techniques.

C. *FTIR NPs CsSU Polymers*

The NPs CsSU powder from the centrifugation process was dried in an oven at 70^0C for 6 hours. The samples obtained were then made into Fourier Transform Infrared (FTIR) (Nicolet™ iS™ 5 TFS Inc.) to obtain a qualitative analysis of organic compounds and NPs CsSU polymer compounds. Samples were prepared by mixing 2mg of chitosan SU dry powder with 100mg KBr to form pellets. The obtained spectrum was compared with standard SU chitosan powder and Tween-80 **(Martins *et al.*, 2012)**.

D. *Morphology NPs CsSU polymers*

The CsSU nanoparticle (NP) powder obtained from the centrifugation process was immediately suspended in 20 mL of solvent and then dried in an oven at 70 °C for 6 hours. The samples obtained were then subjected to morphological observations using SEM image analysis (Shimadzu, SS 550) in the electron beam voltage acceleration of 10-15 kV. The average particle diameter was calculated by analyzing each image with a certain magnification using the Meter© software, version 1.1 with the threshold of differentiation determined according to the scale of the image **(Martins *et al.*, 2012)**.

E. *Zeta potential of NPs CsSU suspension*

The stability of NPs CsSU suspensions was investigated based on the zeta potential using the Delsa™ Nano Series instrument using the electrophoretic light scattering technique. Nanoparticles suspension is considered stable if their zeta potential is around ±30 mV. Particles size distribution of the suspension was investigated using particle size analyzer using Horiba SZ-100 **(Ray *et al.*, 2018)**.

F. *X-Ray diffraction NPs CsSU*

The NPs CsSU powder from the centrifugation process was dried in an oven at 70^0C for 6 hours. The samples obtained were then entered into a Wide-angle X-ray diffraction analysis using a Cu diffractometer at 40 kV, 30 mA (Rigaku Corp; Japan) to observe the crystallinity of NPs CsSU. The spectrum was collected at a scanning speed of 1% minutes with a scanning angle from 2^o to 60^o **(Salar & Kumar, 2016)**.

4. Statistical analysis

Data were evaluated by one-way analysis of variance (ANOVA) using SPSS version 24 and significant differences were analyzed by Duncan's Multiple Range Test (DMRT) with $P < 0.05$.

RESULTS AND DISCUSSION

1. Water content and color of NPs CsSU

Moisture content is a critical parameter in determining the quality of chitosan sea urchin nanoparticles (NPs CsSU). Low moisture content helps preserve the integrity of NPs CsSU by inhibiting microbial activity during storage. As shown in Table (1), the moisture content of NPs CsSU ranged from 12.73 to 18.37%. The lowest moisture content was observed in the sample without the addition of the surfactant Tween-80. In contrast, the addition of Tween-80 at concentrations of 30%, 60%, and 90% resulted in significantly higher moisture content, exceeding the chitosan quality standard (≤10%). This suggests that the presence of Tween-80 may interfere with the drying process of NPs CsSU, likely due to its composition, which includes fatty acid esters derived from sorbitol. Tween-80 (polysorbate) is a hydrophilic, non-ionic surfactant composed of 20 oxyethylene units, and it is commonly used as an emulsifying agent in oil-based emulsions.

Table 1. Analysis of water and color analysis of chitosan sea urchin nanoparticle (NPs CsSU)

Sample	Tween-80 concentration (% w / w)	Moisture content (%)	Hunter scale		
			L*	***a****	***b****
NPs CsSU	0	9.73 ±0.2A	88.74 ±1.0C	7.90 ±0.6A	9.13 ±0.4C
	30	14.95 ±0.4B	71.49 ±1.2B	24.39 ±0.5B	6.23 ±0.3A
	60	16.65 ±0.5C	63.57 ±0.4A	27.68 ±0.5B	7.02 ±0.5B
	90	18.37 ±0.7D	60.26 ±1.4A	28.45 ±0.6C	6.77 ±0.7A

The values followed by the same letters are not significantly different from each other ($\alpha = 0.05$).

The changes in the color of the NPs CsSU suspension with and without Tween-80 concentration showed very significant differences, as shown in Table (1). The results showed that the brightness color (L*) of the NPs CsSU with Tween-80 at concentrations of 60 and 90% v/v were 60.26 and 63.57, respectively. These values were quite insignificant differences. However, the brightness at a concentration of 30% Tween-80 and without Tween-80 were 71.49 and 88.74 (% v/v), respectively. Similar to the yellowish level (a*), the higher the concentration of Tween-80, the greater the yellowish value. It was also similar to the redness level (b*) indicating that the higher the concentration of Tween-80, the smaller the value. Thus, the color may be due to the nature and characteristics of the additives or Tween-80 since it contains fat. In general, the color of chitosan depends on the raw material and the deacetylation process. Alkaline treatment over a prolonged period typically produces chitosan ranging in color from cream to brown, with varying levels of brightness and brightness index values. Similarly, the chroma value of chitosan from the raw material of lobster shells may due to the extraction process and their water content **(Sayari *et al.*, 2016)**.

2. Particle size analysis of NPs CsSU

Fig. (1) shows the size distribution of SU/Tween-80 chitosan nanoparticle prepared with various concentrations of Tween-80. a) 0 (% v/v), b) 30 (% v/v), c) 60 (% v/v), and d) 90 (% v/v). The average particle size (d_{50}) was in the range of 75 - 528 nm. The lowest polydispersity index (PI = 0.72) was obtained at concentration of Tween-80 60 (% v/v) (Fig. 1c), and without dispersing agent the polydispersity index (PI) was 1.42 (Fig. 1a). The shift of the distribution peak to a smaller size indicates that the NPs CsSU in suspension were well-dispersed. The addition of Tween-80 as a dispersing agent during the bead milling process helped prevent nanoparticle agglomeration, thereby reducing particle size distribution and promoting the formation of spherical particles. Moreover, the appropriate concentration of Tween-80 enhanced the binding strength of the chitosan matrix in the composite **(Du *et al.*, 2009)** and improved the suspension's stability **(Gribb & Banfield, 1997)**. Additionally, the high rotational speed during the bead milling process, along with other agitation methods, is believed to contribute to the uniformity of the particle size distribution **(Nesalin & Smith, 2013)**.

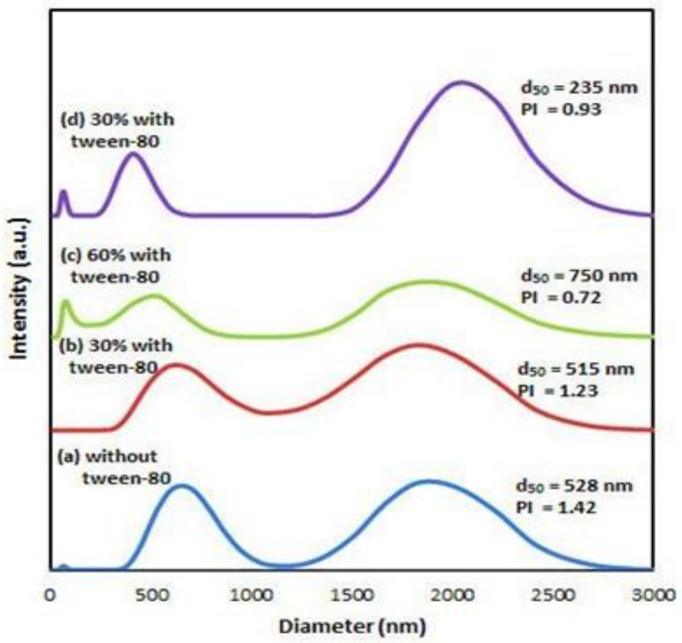


Fig. 1. A) PSA Spectrum Polymer NPs CsSU Beads-Milling Method (a) without Tween-80, (b) 30% with Tween-80, (c) 60% with tween-80, (d) 90% with Tween-80

3. FTIR NPs CsSU polymers

The FTIR spectrum provides a description of the functionalized surface of the NPs CsSU at various concentrations dispersing agent Tween-80 (Fig. 2). The NPs CsSU FTIR spectrum, as shown in Fig. (2e) indicate that NPs CsSU possed a relatively high deacetylation rate (85% DD). The FTIR spectra of NPs CsSU showed strong peaks in the wavelength range of 3480- 3400cm^{-1} which were associated with vibrations that bind to hydrogen (O-H) bonds. In addition, there is an overlap in the same peak region in type 2 amides and primary amine groups from above the N-H region (NH_2). While the peak area in FTIR Tween-80 has the highest spectrum of 3450cm^{-1}. Whereas the peak of the asymmetric- stretching area (C-O-C) at the peak of 1033 cm^{-1} and CH at the peak of 1418cm^{-1} belongs to the C-N vibrational strain at its peak (Fig. 2a-e). In Fig. (2), the peaks of the NPs CsSU FTIR at the peaks of the 3480cm^{-1} shifted to 3255cm^{-1} with increased intensity, also for the hydrogen bonds, they increased as concentrations of NPs CsSU increased.

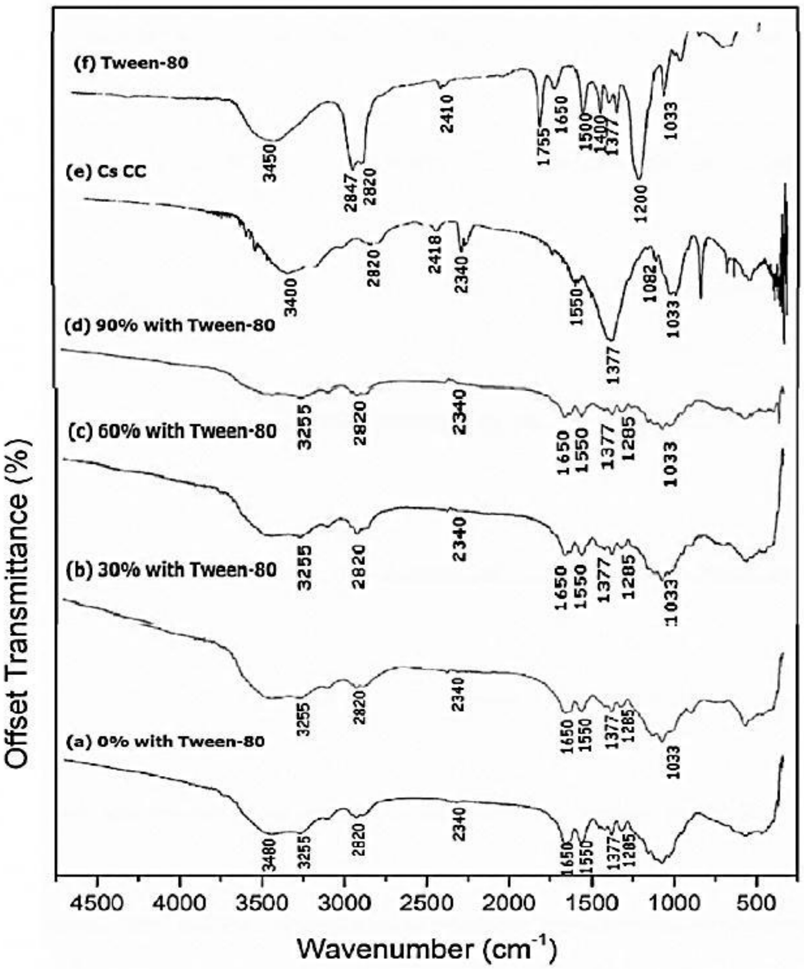


Fig. 2. FTIR Spectrum NPs CsSU Beads-Milling Method (a) without Tween-80, (b) 30% with Tween-80, (c) 60% with Tween-80, (d) 90% with tween-80, (e) Cs CC, (f) Tween-80

All NPs CsSU suspensions showed a weak peak of the amine group C = O (1650cm^{-1} and while the carbonyl group of the 2nd stretching amide at 1550cm^{-1} shifted to 1377cm^{-1}. Similarly, the crosslinked NPs CsSU showed the peak of the C-O (CH_2) stretching symmetry region at the same 2,820cm^{-1} received from CsSU suspension with tween-80 area (Fig. 2a-e). The characteristic C-N peak usually found at 1265cm^{-1}, in case of NPs CsSU suspension contain tween-80 was found at the peak of 1200cm^{-1} which belongs to the saccharide structure. However, this peak was shifted to 1377cm^{-1} in NPs CsSU suspension, without dispersing agent.

Fig. 2(c) shows a shift in the NH_2 functional group, indicating ionic interactions between the chitosan derived from sea urchin (SU chitosan) and the Tween-80 molecules, leading to the formation of NPs CsSU polymer. This shift suggests the formation of cross-links between Tween-80 and the SU chitosan suspension, which occurs as a result of ionic gelation with TPP (tripolyphosphate). The FTIR spectra for all treatments display a characteristic peak around 1650cm^{-1}, corresponding to N–H bending (amide I) vibrations. This peak is absent in the untreated CsSU sample, implying that cross-linking between the amino groups of chitosan and aldehyde groups (possibly from Tween-80 or degradation byproducts) has occurred, forming covalent bonds and altering the original functional group signatures.

4. Morphology NPs CsSU polymers

Fig. (3) shows the morphology of NPs CsSU polymers with varying Tween-80 concentrations (0, 30, 60 and 90 (% v/v)) which were observed using SEM. The results demonstrated that NPs CsSU polymers synthesized with Tween-80 exhibited more uniform morphology and smaller particle sizes. Among the concentrations tested, a Tween-80 concentration of 60% (v/v) produced the smallest and most uniform particles, with an average size below 100 nm (Fig. 3c). In contrast, at Tween-80 concentrations of 30 and 90% (v/v), the particle sizes were more variable, and particle agglomeration was observed (Figs. 3b, d). On the other hand, NPs CsSU prepared without Tween-80 showed relatively small particle sizes, though in limited quantities, and displayed a highly homogeneous morphology (Fig. 3a).

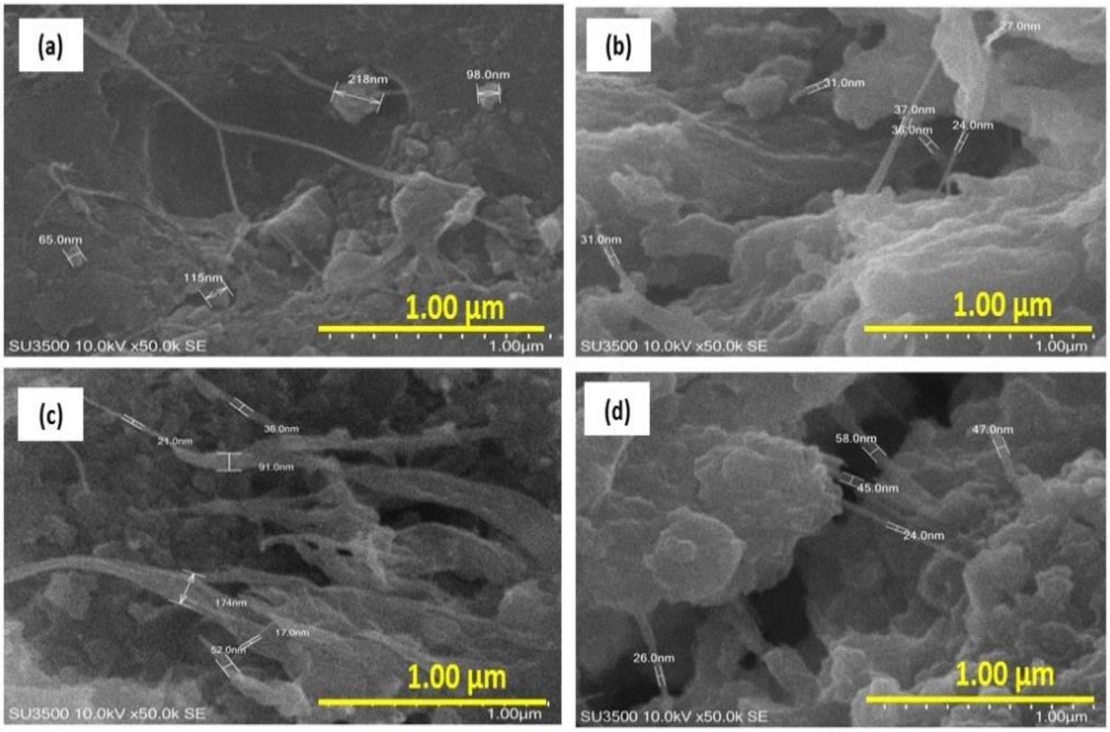
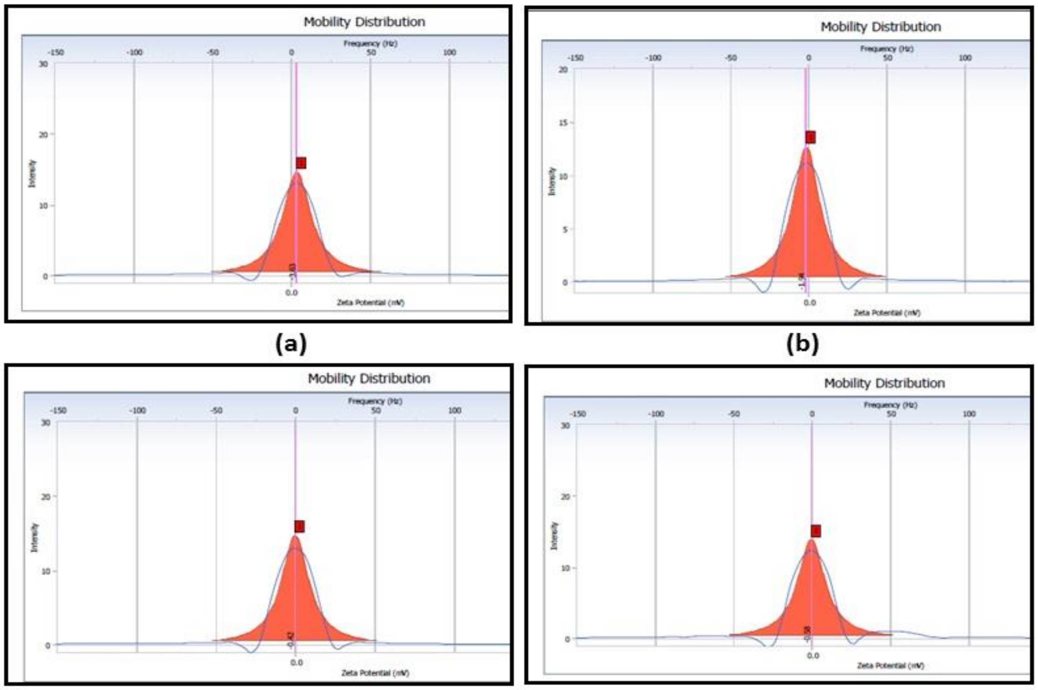


Fig. 3. SEM-morphology polymer NPs CsSU beads-milling method (a) without Tween-80, (b) 30% with Tween-80, (c) 60% with tween-80, (d) 90% with Tween-80.

The particle size reduction of NPs CsSU polymers using the bead milling method effectively produces a more homogeneous particle distribution. Additionally, the incorporation of surfactant (Tween-80) and tripolyphosphate (TPP) enhances the mechanical properties of CsSU, which is naturally brittle, by promoting the formation of ionic cross-links between chitosan molecules. In this process, the CsSU suspension is mixed with 0.003% (w/v) TPP and varying concentrations of Tween-80. Tween-80 acts as a surfactant to stabilize the particle emulsion during bead milling, preventing agglomeration and facilitating the formation of NPs CsSU polymers. Furthermore, the surfactant helps coat and stabilize the chitosan particles in solution, making the size reduction process more effective and minimizing particle aggregation **(Xu & Du, 2003)**.

5. Zeta Potential of NPs CsSU Suspension

The zeta potential of the NPs CsSU polymer suspension is shown in Fig. (4), showing that the average treatment values of each Tween-80 were 1.94, 3.63, 0.58, and 0.42 mV and only formed 1 peak (Fig. 4). Therefore an increase in the concentration of tween-80 can reduce the zeta value as a form of an increase in ion concentration in the NPs CsSU medium is very significant (Figs. 4c and d). Whereas a decrease in Tween-80 concentration can increase the zeta value (Fig. 4b) and the sample without the addition of tween-80 low zeta value. Therefore the presence of an ionic environment in a colloid will have an effect on the observed zeta potential. Likewise, the use of Tween-80 surfactant as a dispersing medium is able to show the interaction on the surface of the particle and is able to stabilize the state of dispersion in the coating media, namely CsSU. On the contrary, the results of this study prove that the presence of electrostatic stabilization in the crab shell chitosan suspension **(Rochima *et al.*, 2017)**.



(a) **(b)**

(c) **(d)**

Fig. 4. Potential zeta polymer NPs CsSU beads-milling method (a) without Tween-80, (b) 30% with Tween-80, (c) 60% with Tween-80, (d) 90% with Tween-80

Based on the zeta potential measurements shown in Fig. 4(a, b), an increase in zeta potential was observed, whereas in Fig. (4c, d), a decrease in zeta potential occurred alongside a reduction in the Z-average diameter of the NPs CsSU particles produced through the bead milling process with varying concentrations of surfactant. Zeta potential serves as an indicator of the repulsive forces between particles and can be used to predict the stability of a colloidal dispersion system. These results suggest that the chitosan nanoparticles produced via bead milling remain unstable in the dispersion, as indicated by their tendency to aggregate and form clusters. The relatively low zeta potential values across all samples may be influenced by several factors, including the physical stability of the dispersion as explained by the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory, the type and concentration of the surfactant or dispersing agent used, and the pH of the dispersion medium **(Joni *et al.*, 2017)**.

6. X-ray diffraction NPs CsSU

Fig. (5) shows the XRD spectra of SU/Tween-80 chitosan nanoparticle polymers prepared with varying concentrations of Tween-80. The XRD pattern of the NPs CsSU polymer displays a diffraction peak at 2θ = 19.05° (Fig. 5c), which corresponds to a characteristic peak of chitosan. Notably, this peak is slightly shifted to a lower angle compared to the typical chitosan peak, which is usually observed around 2θ = 26.16°. This shift can be attributed to interactions between chitosan and Tween-80 during the formation of the NPs CsSU polymer, indicating possible structural modifications or reduced crystallinity due to the incorporation of the surfactant.

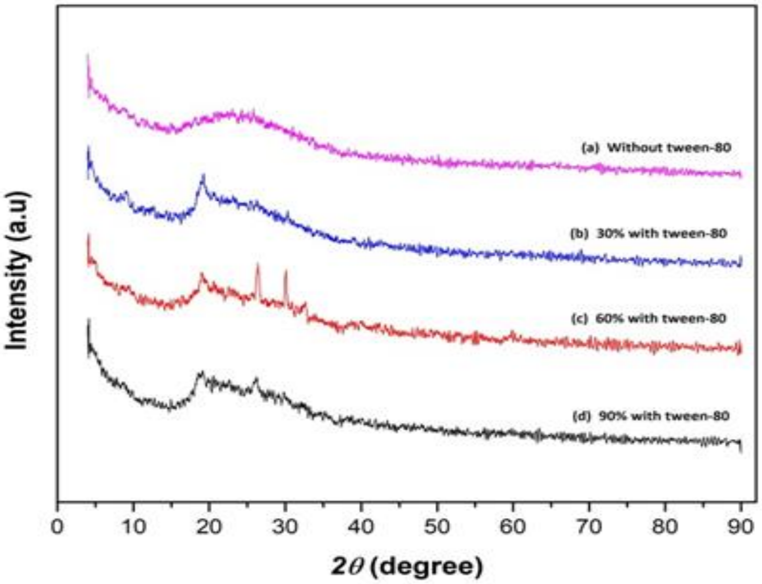


Fig. 5. XRD spectrum polymer NPs CsSU beads-milling method (a) without Tween-80, (b) 30% with Tween-80, (c) 60% with Tween-80, (d) 90% with Tween-80

The lower intensity of the diffraction peaks in the XRD patterns of NPs CsSU polymers, particularly at a Tween-80 concentration of 60% (v/v), indicates a more amorphous structure. This is further supported by the appearance of additional peaks at $2\theta = 26.16°$, along with an increase in intensity at $2\theta = 19.05°$ observed at higher Tween-80 concentrations. These changes suggest a transition from an amorphous to a more crystalline form with increasing surfactant concentration. Additionally, a weak peak at $2\theta = 29.78°$ was observed in the NPs CsSU/Tween-80 samples. These structural modifications reflect the formation of intramolecular networks between chitosan, TPP, and Tween-80. The interaction between these components alters the molecular arrangement, reducing the flexibility of the polymer chains and promoting partial crystallinity within the nanoparticles (Thakur & Taranjit, 2011).

CONCLUSION

Characterization of chitosan ion interactions with Tween-80 showed an influence in the formation of chitosan sea urchin nanoparticle (NPs CsSU) polymers due to the use of surfactant media in chitosan suspensions using a beads-milling method. Therefore, it can be concluded that the formation of NPs CsSU polymers is influenced by the use of surfactant media that is at the concentration of Tween-80 (60% v/v) and gives an effect on smaller particle size and low polydispersity index, and the morphology of the average particle size below 100 nm with lower crystallinity values in the form of amorphous.

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