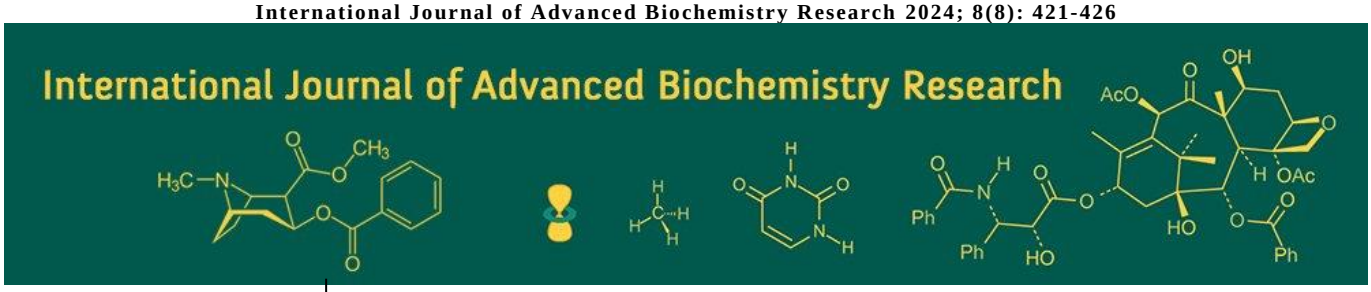

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Synthesis and characterization of chitosan nanoparticles: Insights from *in-vitro* analysis

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Abstract
Chitosan nanoparticles (CNPs) are widely acknowledged for their versatility in various domains, particularly in areas relevant to pharmaceuticals and vaccines. This study aimed to produce CNPs as a foundational element for nanoparticle-based delivery systems intended for potential use in vaccine development. We outline a straightforward yet effective synthesis method for generating high-yield, uniform CNPs ranging in size from 100 to 250 nm. The synthesis involved ionic gelation using medium molecular weight chitosan (with a deacetylation level exceeding 75%) and sodium tripolyphosphate as a crosslinker. This was followed by thorough homogenization to ensure consistency, and purification via 0.45 µm polyethersulfone syringe filters. The CNPs underwent characterization for zeta size, zeta charge, and polydispersity index (PDI), while scanning transmission electron microscopy was employed for morphological analysis. Internalization of these chitosan nanoparticles into MDBK cells was achieved through the use of FITC-conjugated counterparts (FCNP). Furthermore, we investigated the impact of chitosan concentrations on CNP size, charge, and polydispersity. These synthesized CNPs exhibit a strong affinity for proteins, DNA, and other biomolecules, positioning them as potential candidates for the development of nanoparticle-based delivery systems tailored to vaccine development.

Keywords: Chitosan nanoparticle, sodium tripolyphosphate, fluorescein isothiocyanate, 4',6-diamidino-2-phenylindole, polydispersity index

Introduction
Chitosan, derived from the deacetylation of chitin, is a versatile compound with wide-ranging applications in various fields of science. With its unique properties, including solubility and stability, chitosan has garnered significant attention, particularly in nanotechnology, where it serves as a key material for nanoparticle synthesis. Chitosan nanoparticles, synthesized through methods like ionotropic gelation, microemulsion, polyelectrolyte complexation, emulsification-cross linking, and coprecipitation, offer controlled characteristics such as size and surface charge. These nanoparticles demonstrate promising immunostimulatory effects, enhancing immune responses and antigen presentation [1-3]. They also serve as effective carriers for vaccines and drugs due to their biocompatibility, controlled release kinetics, and easy preparation methods [4-7]. The significance of chitosan nanoparticles extends beyond biomedical applications, as they find utility in various other areas. In parenteral drug delivery, chitosan nanoparticles exhibit altered biodistribution based on their size and surface zeta potential. Larger particles are rapidly taken up by the reticuloendothelial system, while smaller particles experience prolonged circulation [8]. In per-oral administration, chitosan nanoparticles enhance the bioavailability of drugs and biomolecules through muco-adhesion, prolonging absorption time and improving overall efficacy [9]. Additionally, chitosan nanoparticles serve as efficient non-viral gene delivery systems, offering targeted delivery of DNA and siRNA to specific cells [10-13]. Surface modifications further enhance their efficacy, enabling the successful administration and expression of genes in animal models [14]. The preparation methods for chitosan nanoparticles, such as ionotropic gelation, microemulsion, polyelectrolyte complexation, emulsification-cross linking, and coprecipitation, highlight the versatility of this material [15, 16]. Each method leverages chitosan's chemical properties to achieve controlled nanoparticle characteristics, including size, surface charge, and drug loading capacity.

Moreover, the physicochemical parameters, such as pH and temperature, play crucial roles in nanoparticle synthesis, influencing particle size and stability [15, 16]. Ultrasonication aids in achieving uniform dispersion, while phase transitions induced by changes in pH and temperature affect nanoparticle solubility and morphology [17]. In conclusion, chitosan nanoparticles represent a promising avenue for various applications, ranging from drug delivery to gene therapy and beyond. Their versatility, biocompatibility, and ease of preparation make them attractive candidates for further research and development in nanomedicine and related fields. This study aimed to investigate the synthesis of chitosan−STPP nanoparticles using the ionic gelation technique. Following synthesis, the prepared CNP underwent characterization primarily through zeta analysis. The morphological aspect of the nanoparticles was assessed using transmission electron microscopy (TEM). Furthermore, to evaluate their potential for cellular uptake and imaging, the synthesized CNP were conjugated with FITC and subjected to further examination. cellular uptake and imaging by conjugating with FITC

Materials and Methods

In this study, MDBK (Madin-Darby Bovine Kidney) cells were cultured using EMEM (Eagle's Minimum Essential Medium) supplemented with 10% Fetal Bovine Serum (FBS) as the growth media. The cells were treated with trypsin for detachment during passaging. Chitosan Sigma-Aldrich and Sodium Tripolyphosphate (STPP) Sigma-Aldrich were utilized as key chemical components for nanoparticle formulation. Fluorescent labeling and visualization were achieved using Fluorescein Isothiocyanate (FITC) for general labeling, 4′,6-Diamidino-2-phenylindole (DAPI) for nuclear staining, and Rhodamine Phalloidin (ActinRed) from Thermo Fisher Scientific for actin filament visualization.

Preparation of Chitosan and STPP Solutions

The process began by slowly dissolving chitosan in an aqueous solution containing 1M acetic acid while also employing sonication until achieving a transparent solution. Subsequently, the chitosan solution underwent dilution with deionized water, pH was adjusted to 5.0 by adding 7.5% $NaHCO_3$. The prepared solution was filtered through a 0.45 mm filter to yield a 1% final chitosan stock solution (w/v or 10 mg/ml). The chitosan stock solution was then further diluted with deionized water to produce chitosan solutions with varying concentrations: 0.5 mg/ml, 1.0 mg/ml and 1.5 mg/ml. Concurrently, TPP was dissolved in deionized water at concentrations of 10mg/ml and diluted with deionized water to produce a final concentration of 0.5 mg/ml.

Formulation of Chitosan-STPP Nanoparticles (CNP)

Based on the principle of ionic crosslinking, Chitosan-STPP nanoparticles synthesis involves intra and inter-molecular crosslinking interactions between positively charged chitosan and negatively charged STPP molecules. To achieve this, 2.5 ml of STPP solution with a concentration of 0.5 mg/ml was slowly added dropwise to 5 ml of chitosan solution, which varied in concentrations from 0.5 mg/ml to 1.5mg/ml. This process was conducted under constant magnetic stirring at room temperature. Following synthesis, the Chitosan-STPP nanoparticles were separated through centrifugation at 10,000 RCF for 30 minutes at 4 °C, after which the supernatant was discarded. Subsequently, the nanoparticles were washed with distilled water. Upon completion of the washing step, the nanoparticles were resuspended in a PBS solution with a pH of 7.4 and stored at 4 °C until further analysis.

Cellular Uptake study

The synthesis of fluorescein isothiocyanate (FITC)-labelled chitosan was achieved through a reaction involving the isothiocyanate moiety of FITC and the primary amino group of chitosan. In brief, 100 mg of FITC dissolved in 150 ml of anhydrous methanol was added to a solution containing 100 ml of 1% chitosan in 0.1 M acetic acid. The reaction proceeded for 3 hours under dark conditions at room temperature. Subsequently, the FITC-labelled chitosan was precipitated by adjusting the pH to 8-9 using 0.5 M NaOH. To eliminate any unconjugated FITC, the precipitate underwent several rounds of washing and centrifugation (at 40,000 g for 10 minutes) until no fluorescence was observed in the supernatant. The resulting fCS was then dissolved in 80 ml of 0.1 M acetic acid. FITC-labelled chitosan nanoparticles (FCNP) were formed by adding 2 ml of a tripolyphosphate (TPP) solution (0.28 and 0.43 mg/mL) to 5 ml of FITC-labelled chitosan solution (1 mg/ml) while stirring on a magnetic stirrer at room temperature. Immediately following preparation, the dispersions of FCNP were characterised and employed for uptake studies. The particle size and zeta potential of the nanoparticles were determined in triplicate by dynamic light scattering method (Nanotrac Wave II, Japan).

Pathway Identification for Nanoparticle Internalization

To explore the mechanisms underlying the intracellular uptake of CNP, MDBK cells were seeded in 6-well plates and subsequently treated with sucrose, a known chemical inhibitor for 0.5 hours at 37°C [18, 19]. After this pretreatment, FITC-CsNPs at a concentration of 1.5 mg/mL were introduced to the cells. The effects of sucrose on nanoparticle (NP) uptake were then meticulously examined using confocal microscopy. The sucrose was applied at a concentration of 450 mM, based on previous reports indicating its efficacy in inhibiting both phagocytosis and clathrin-mediated endocytosis [19]. This setup allowed for a detailed analysis of how the inhibition of these pathways impacts the intracellular uptake of the nanoparticles, providing valuable insights into the cellular mechanisms at play.

Transmission Electron Microscopy

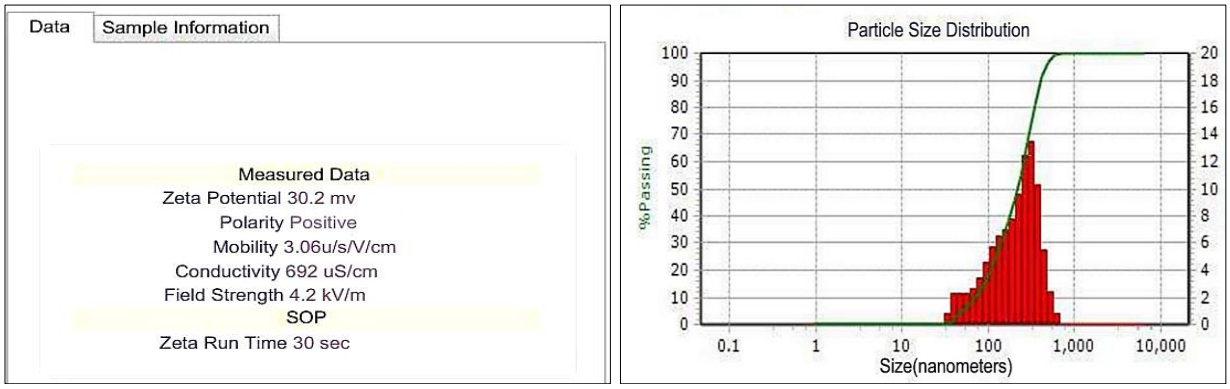
A Talos™ F200X G2 scanning transmission electron microscope (STEM) with scanning transmission electron microscopy (STEM) served as the tool for examining the morphology of nanoparticles. The colloidal suspension containing chitosan nanoparticles underwent a 2 minute sonication to ensure uniform dispersion. Following this, a singular drop of the colloidal suspension was uniformly spread onto a carbon-coated copper grid which was negatively stained with uranyl acetate and allowed to air-dry at ambient conditions and images were capture at varied magnifications.

Results

Preparation and Optimization of Chitosan-STPP Nanoparticles

Chitosan nanoparticles were successfully prepared using the ionic gelation method with varying concentrations of chitosan (1.5 mg/ml) and a constant concentration of sodium

tripolyphosphate (STPP) at 0.5 mg/ml. The formulations resulted in nanoparticles with distinct sizes and zeta potentials, as summarized in Table 1. The mean diameter of the chitosan nanoparticles varied depending on the concentration of chitosan in the formulation. At a chitosan concentration of 0.5 mg/ml, the nanoparticles had a mean diameter of 175 nm. Increasing the chitosan concentration to 1 mg/ml resulted in larger nanoparticles with a mean diameter of 236 nm. Further increase in chitosan concentration to 1.5 mg/ml led to the formation of even larger nanoparticles, with a mean diameter of 374 nm. The zeta potential and PDI of the chitosan nanoparticles did not show significant variation with changes in chitosan concentration.



A B

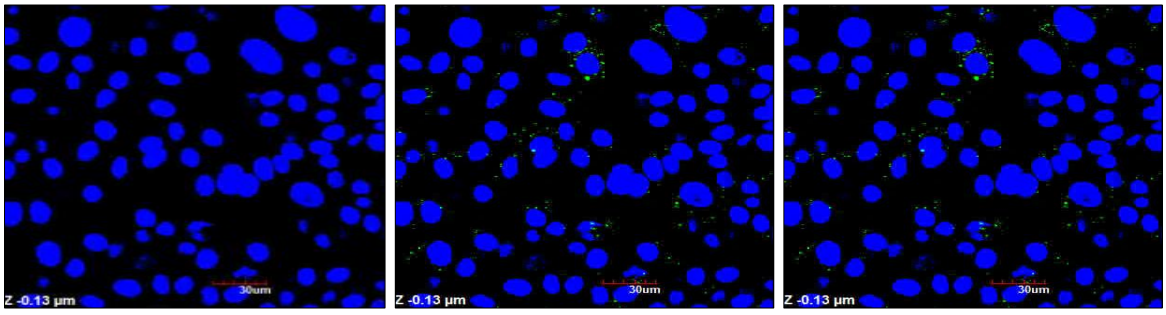
Fig 1: A. zeta potential in mV, B. Zeta size distribution. All measurements were recorded in triplicate, along with their respective standard deviations.

Table 1: The table presents results from zeta analysis of chitosan nanoparticles across three different chitosan concentrations, each paired with a constant

Sr. No.	Chitosan mg/ml (A)	STPP mg/ml (B)	Ratio (A:B)	Zeta Size (nm)	PDI	Zeta Potential (mV)
1	0.5	0.5	2:1	175±50	0.3±0.2	30.2±3.6
2	1.0	0.5	4:1	236±86	0.4±0.6	33.8±2.1
3	1.5	0.5	6:1	374±27	0.9±0.3	32.6±1.7

Cellular Uptake of CNP Conjugated with FITC

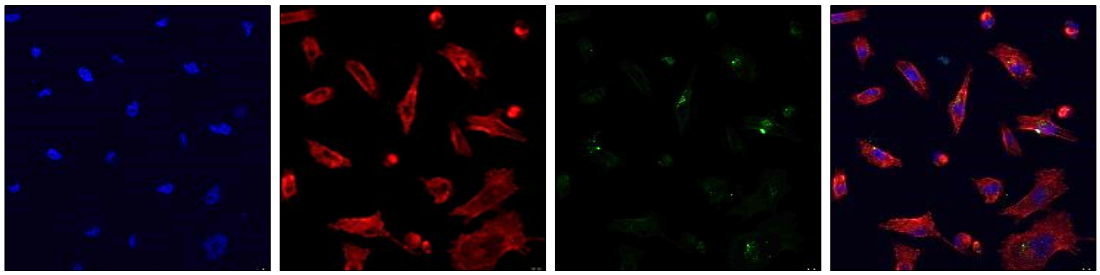
The quantitative analysis of the FCNP uptake by MDBK cells is illustrated in Fig 2 C. This demonstrates a pronounced internalisation pattern at a depth of Z-0.13 µm. Confocal microscopy images corroborated these findings, revealing the presence of FCNP within the cytoplasm of MDBK cells, with a notable accumulation around the nucleus.



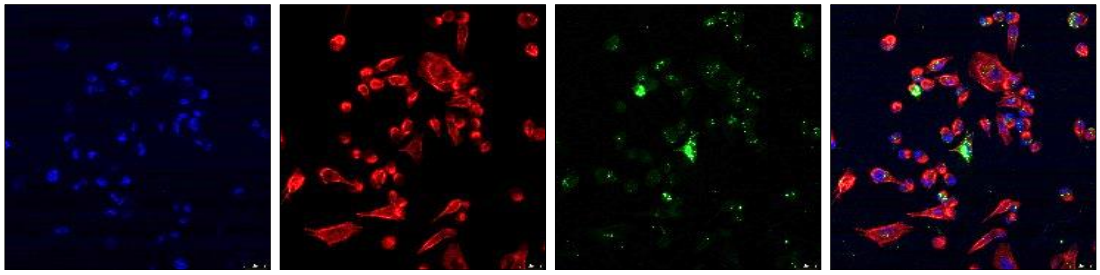
A B C

Fig 2: Confocal imaging was employed to visualize the uptake of FITC-conjugated chitosan nanoparticles within MDBK cells, specifically observed at a depth of 0.13 µm along the z-axis. A. DAPI filter B. FITC filter C. merged image.

Cellular Internalization Pathways



A1 A2 A3 A4



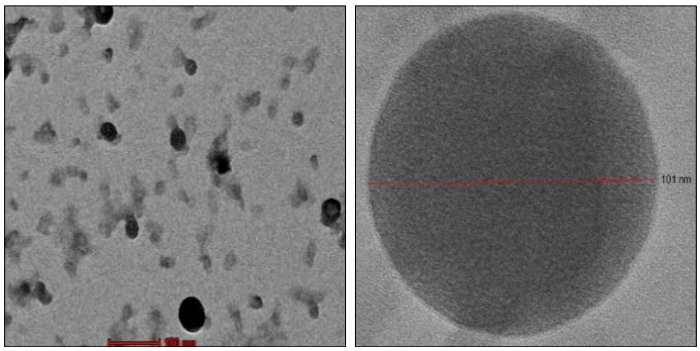
B1 B2 B3 B4

Fig 3: Pathway Analysis of FITC-Chitosan Nanoparticle Uptake in MDBK Cells, using different stains with their respective filters i.e. (DAPI, TRITC, FITC, Merged) from left to right A. MDBK cells treated with sucrose 450 mM and subsequently FCNP. B. MDBK cells treated with FCNP.

In this study, we investigated clathrin-mediated endocytosis and phagocytosis as potential mechanisms for the uptake of FCNPs by MDBK cells. To explore these pathways, sucrose was employed as a chemical inhibitor.The results, illustrated in Figure 3, revealed that the internalization of CNPs in MDBK cells was diminished in the presence of sucrose, as shown in Figure 3A, compared to the untreated control group depicted in Figure 3B. This reduction in CNP uptake suggests that FCNPs were taken up more efficiently in the absence of sucrose inhibition.

Morphological Characterization by TEM

In this study, tem (TEM) images have depicted the morphological characteristics and surface features of the nanoparticles. These particles exhibit a predominantly spherical shape, with a smooth surface texture, and a size of approximately 100 nanometers (Figure 3).



A B

Fig 4: Transmission electron microscopy (TEM) images of chitosan nanoparticles were captured at two different magnifications, with an electron beam energized at 200 kilovolt acceleration. Thus revealing their spherical shape and uniform distribution. A. 700x magnification scale bar 500 nm B. 1400x magnification scale bar 50nm.

Discussion

Chitosan nanoparticles were synthesised using the ionic gelation technique, with a focus on optimizing the concentrations of chitosan and TPP for achieving nano-scale size [20]. The properties of these nanoparticles were found to impact their biological performance. Chitosan contains amino groups which can become protonated in low pH conditions, enhancing its solubility in acidic solutions [21]. TPP acts as a cross-linking agent, possessing a negative charge [22]. The interaction between positively charged chitosan and negatively charged TPP leads to the formation of CSNPs. The size of these nanoparticles is largely influenced by the concentrations of chitosan and TPP solutions, with higher concentrations resulting in larger nanoparticle sizes. We conducted the synthesis of chitosan nanoparticles (CNPs) by modulating the concentrations of chitosan, while ensuring that the concentration of sodium tripolyphosphate (STPP) remained constant throughout the process [23]. The observed variation in nanoparticle size with changing chitosan concentration underscores the importance of formulation parameters in nanoparticle synthesis. The mean diameter of the chitosan nanoparticles exhibited a clear trend, increasing as the chitosan concentration was raised from 0.5 mg/ml to 1.5 mg/ml. This relationship between chitosan concentration and nanoparticle size suggests that chitosan plays a crucial role in nanoparticle formation, potentially influencing nucleation and growth processes during ionic gelation [22]. The augmentation in particle size resulting from higher concentrations of chitosan may be ascribed to the greater spatial separation among chitosan molecules at elevated concentrations, leading to the formation of larger particles. Conversely, lower concentrations of chitosan yielded smaller particle sizes due to reduced viscosity during ionic gelation. This observation resonates with the conclusions drawn by [22], providing further support for the relationship between chitosan concentration and nanoparticle size as demonstrated in their research. This observation suggests a nuanced interplay between the chitosan and STPP constituents, wherein alterations in chitosan at concentration might not exert a pronounced influence on the zeta potential of the nanoparticles within the parameters tested. Our findings underscored the heightened sensitivity of the nanoparticles' zeta potential to shifts in pH and ionic concentration within the solution milieu. This sensitivity is a hallmark characteristic of chitosan-based systems, attributed primarily

to the protonation and deprotonation dynamics of the amino groups decorating the nanoparticle surface. Notably, our investigation corroborates existing literature indicating that at a pH of 5, chitosan nanoparticles tend to exhibit a markedly positive zeta potential [24]. This positively charged state imparts considerable stability to the nanoparticles by engendering electrostatic repulsion forces between individual particles [25].

The cellular uptake study demonstrated the efficient internalization of chitosan nanoparticles conjugated with FITC by MDBK cells. The quantitative analysis revealed a pronounced internalization pattern, with confocal microscopy images confirming the presence of nanoparticles within the cytoplasm, particularly around the nucleus. This robust cellular uptake underscores the potential of chitosan nanoparticles as effective carriers for intracellular delivery of therapeutic agents and imaging probes [26]. Moreover, the accumulation of nanoparticles around the nucleus suggests their ability to target specific cellular compartments, which could be exploited for targeted drug delivery and imaging applications [27]. Our findings regarding the internalization of chitosan FITC nanoparticles align closely with those of previous studies [28] The internalization of nanoparticles into cells is governed by various factors, including nanoparticle size, surface chemistry, and cellular uptake mechanisms [29]. The observed cellular uptake of chitosan nanoparticles highlights their favourable characteristics for intracellular delivery and underscores their potential in biomedical applications. Figure 3A illustrates that treatment with sucrose effectively blocked cellular uptake, leading to a significant reduction in internalization within 0.5 hours compared to the control group. This finding suggests that in addition to clathrin-mediated endocytosis, the uptake of FITC-CsNPs is also influenced by the phagocytosis pathway. For the uptake of solid particles, phagocytosis emerges as a prominent mechanism,36 in conjunction with the classical pathways of clathrin-mediated [30-31] and caveolae-mediated endocytosis [32-33]. The caveolae-mediated pathway is especially effective for particles under 70 nm in diameter, due to the size range of caveolar endocytic vesicles (40-70 nm). As a result, this study explored clathrin-mediated endocytosis and phagocytosis as potential mechanisms for FCNP uptake in MDBK cells. Research indicates that adding hypertonic sucrose to the medium can cause clathrin microcages to form on the plasma membrane's inner surface, reducing the availability of clathrin required for normal coated pit assembly [34]. As a result, our findings suggest that clathrin is the primary mediator for the uptake of FITC-CsNPs, although alternative pathways may also be involved. Furthermore, while phagocytosis is known to facilitate the uptake of nanoparticles larger than 70 nm, it is notably the dominant mechanism for nanoparticle internalization in MDBK cells

Morphological characterization of the nanoparticles by transmission electron microscopy (TEM) provided further insights into their structural properties [35]. The TEM images revealed nanoparticles with a predominantly spherical shape, smooth surface texture, and uniform size distribution of approximately 100 nanometers. The images we observed closely resemble those depicted in numerous reports found in the literature [36]. These morphological characteristics are desirable for biomedical applications as they contribute to enhanced stability, biocompatibility, and cellular uptake. The spherical shape of the nanoparticles minimizes nonspecific interactions with biological components and facilitates their uptake by cells [37]. The smooth surface texture indicates a uniform coating of chitosan and STPP, which is essential for maintaining nanoparticle stability and preventing aggregation. The uniform size distribution further confirms the reproducibility of the synthesis method and suggests tight control over nanoparticle size, which is crucial for achieving desired pharmacokinetic and biodistribution profiles *in vivo*.

Conclusion

Overall, the successful synthesis and characterization of chitosan-STPP nanoparticles highlight their potential as a promising candidate for vaccine and drug delivery systems. Further research and development in this area could focus on optimizing nanoparticle formulations for specific vaccine antigens, evaluating their immunogenicity and efficacy in preclinical models, and exploring additional functionalization strategies to enhance their performance. With continued innovation and investigation, chitosan nanoparticles could emerge as a versatile and effective platform for the delivery of vaccines, contributing to advancements in preventive medicine and public health. In conclusion, chitosan nanoparticles represent a promising avenue for various applications, ranging from drug delivery to gene therapy and beyond.

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