1 **Preparation of Nanoscale *Bacillus thuringiensis* Chitinases Using**
2 **Silica Nanoparticles for Nematicide Delivery**

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Abstract

A series of amino, carboxylic, and aldehydic surface-grafted silica nanoparticles (SNPs) was prepared based on SiO₂ NYSi40 nanoparticles to develop an efficient, biocompatible, and cost-effective biopesticide delivery system. *Bacillus thuringiensis* chitinase (Chi9602) was immobilized onto SNP surface to prepare nanoscale chitinases (SNPCs) through electrostatic adsorption and covalent binding. The specimens were characterized by Fourier transform infrared, scanning electron microscopy, and zeta-potential analyses. The delivery capacity of the SNPs in *Caenorhabditis elegans* N2 was observed by immunofluorescence. Results demonstrated that amino-grafted SiO₂ nanoparticles with Chi9602 electrostatically adsorbed onto their surface (SNPC2) exhibited a relatively high enzyme immobilization rate (80.2%) and the highest (94.1%) residual enzyme activity among all SNPCs. SNPC2 also showed wider pH tolerance and relatively higher thermostability and ultraviolet radiation resistance capacity than Chi9602. Bioassays further showed that SNPC2 synergistically enhanced the nematocidal effect of *B. thuringiensis* YBT-020 preparation against *C. elegans*, with a reduced LC₅₀ of 8.35 mg/mL and a shortened LT₅₀ of 12.04 h. Immunofluorescence assays showed that SNPC2 had considerable delivery capacity to carry a large protein into *C. elegans*. Therefore, SNP2 can serve as an efficient nanocarrier for the delivery of macromolecular proteic biopesticides or drugs, indicating potential agricultural or biotechnological applications.

Key words: Silica nanoparticles; Surface grafting; Chitinase; Nanocarrier; Biocontrol agent

38 **Abbreviations**

NPs	Nanoparticles
SNPs	Silica nanoparticles
SNP1	SiO ₂ NYSi40 nanoparticles
SNP2	Surface amino-grafted SNP1
SNP3	Surface carboxylic-grafted SNP1
SNP4	Surface aldehydic-grafted SNP1
SNPC	Composite nanoscale chitinase with Chi9602 and SNPs
SNPC1	Nanoscale chitinases of Chi9602 electrostatic adsorption on SNP1
SNPC2	Nanoscale chitinases of Chi9602 electrostatic adsorption on SNP2
SNPC3	Nanoscale chitinases of Chi9602 electrostatic adsorption on SNP3
SNPC4	Nanoscale chitinases of Chi9602 covalently linked on SNP3
SNPC5	Nanoscale chitinases of Chi9602 covalently linked on SNP4

1. Introduction

Numerous studies have focused on nanotechnology because nanoparticles (NPs) with dimensions on the order of 100 nm or less often possess a high specific surface area, a strong adsorption capacity, and various specific physicochemical, optical, and electrical properties [1–3]. Nanoloading of NP in nanodrug delivery systems can change membrane-transport mechanisms, increase biological-membrane permeability, and eliminate special bio-barriers, thereby promoting direct drug diffusion and delivery to intracellular targets [4, 5]. Therefore, a nanodrug delivery system can improve the utilization rates and curative effects and decrease the side-effects of a drug. Accordingly, such systems have been extensively developed and used in various biomedical fields, including cancer treatment, diagnostic reagent development, and gene therapy [6–8]. However, the problems of relatively high cost and environmentally unfriendly nature seriously limit the agro-biotechnological applications of the most commonly used NPs, such as gold-, iron oxide-, or ZnO-like NPs [3, 5, 9], which often require a great quantity and direct environmental exposure when they are used to prepare the biopesticides.

Recently, numerous studies have focused on silica nanoparticles (SNPs) use in pesticides [10]. They have been shown to be high stability, chemical versatility, biocompatibility, and low cost have potential as nano-pesticide delivery system for agro-biotechnological applications [11–13]. Adsorption of α -pinene and linalool onto SNPs resulted in an effective formulation that enhanced the antifeedant potential of individual terpenes against insects [13]. The porous hollow silica nanoparticles (PHSN) carriers remarkably improved the photostability of avermectin by entrapping it into the hollow core of the nanoparticle carriers [14]. However, while SNPs have witnessed a remarkable increase in research on agricultural applications, the

64 investigations of the feasibility of certain SNPs used for efficient delivery of the
65 proteic biopesticides are limited. Therefore, studies that focus on cost-effective and
66 practical nano-biopesticide delivery systems to control agricultural and forestry pests
67 are still necessary.

68 The misuse of chemical pesticides has been known to cause significant
69 environmental threat and serious loss of biodiversity [3, 11]. One feasible approach to
70 mitigate such problems is the use of biopesticides as an alternative means to control
71 agricultural and forestry pests [3]. Chitin is one of the most common natural
72 macromolecules and is widely present in cell walls of fungi, peritrophic membranes of
73 insect, egg shells of nematodes, and so on [15–17]. Chitinase is a glycosyl hydrolase
74 that can catalyze the hydrolysis of β -1,4-glycosidic bonds of chitin to form
75 *N*-acetyl-D-glucosamine [18, 19]. To utilize such hydrolysis, a number of researchers
76 have demonstrated that chitinases possess biocontrol capacity as biopesticides
77 [20, 21]. In addition, chitinase can be used as a synergetic biocontrol agent in *Bacillus*
78 *thuringiensis* preparations, which have been proven to be relatively highly
79 insect-specific, remarkably safe for non-target fauna, and cost-effective for
80 agricultural biocontrol [22–24]. In practical applications, many enzymes have delayed
81 action and are sensitive to the environment, which can obviously reduce their utility
82 [25]. Nanopesticide delivery systems that possess NP-related properties have a
83 potential in agro-biotechnological applications because of their capacity to alleviate
84 these problems [11, 26–28].

85 NP preparation is involved in size reduction to nanoscale levels via top-down
86 methods, such as milling, high-pressure homogenization, or sonication; whereas
87 bottom-up processes involve reactive precipitation and solvent displacement [1]. In
88 addition, most NPs require surface modification and grafting of specific chemical

groups to become excellent nanocarriers. Therefore, enzymes and proteins can be immobilized on the NP surface through grafting with these specific chemical groups to achieve excellent nanoscale properties, such as efficient and stable delivery capacity.

The current study aims to develop efficient and stable SNPs for use as a chitinase delivery system in biocontrol applications. The surface groups of hydroxylated nano SiO₂, SiO₂ NYSi40, were initially chemically modified through amination, carboxylation, and hydroformylation to prepare several aminated, hydroxylated, carboxylated, and hydroformylated SNPs. Subsequent Fourier transform infrared (FTIR) spectroscopy and zeta-potential analyses revealed that *B. thuringiensis* chitinase Chi9602 was loaded onto the five SNPs via electrostatic adsorption or covalent linkage. The chitinase immobilization rate and specific chitinase activity of the various SNPCs were comparatively determined. The enzymatic stability of an optimized SNPC2 under different temperature, ultraviolet radiation, and pH conditions was investigated. Moreover its efficiency at Chi9602 delivery in *C. elegans* larvae was evaluated in laboratory trials.

2. Materials and methods

2.1. Materials

Nano SiO₂ NYSi40 (99.9% purity; average particle size = 40 nm) was purchased from Beijing Boyu High-tech New Materials Technology Co., Ltd. (Beijing, China). (3-aminopropyl) triethoxysilane (APTES) was purchased from Yao Hua Chemical Plant (Shanghai, China); *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Jili Biochemical Co. (Shanghai, China); and *N*-hydroxysuccinimide (NHS) was purchased from Shanghai Bo Biological

Technology Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) was purchased from Wuhan Bai Wei Nuo Biological Technology Co., Ltd. (Wuhan, China). All other reagents were analytical grade.

2.2. Preparation of *B. thuringiensis* chitinase Chi9602

The chitinase gene *chi* (GenBank Accession: KF671757.1) was derived from *B. thuringiensis* subsp. *tenebrionis* wild-type strain YBT-9602 (Microbial Genetic Stock Center, Wuhan, China). The previously constructed recombinant plasmid pMB332 [29], which harbors the *chi* coding sequence (codons 35 to 676), was used to express the Chi9602 protein in *Escherichia coli* JM109 cells, by following the procedures described previously [29]. The expressed chitinase Chi9602 was further purified using a Ni-NTA purification kit (Seven Sea Biological Technology Co., Ltd., Shanghai, China), which was followed by an ultrafiltration step at 10 kDa pore size to remove the imidazole compounds and smaller molecules. The collected Chi9602 was detected using SDS-PAGE by following the method described by Laemmli [30].

2.3. Chitinase specific activity analysis

For preparation of 2% colloidal chitin, a small amount of deionized water was added to 2 g of chitin, which was then ground into fine powder for approximately 10 min. The fine chitin was completely dissolved in 45 mL of pre-cooled concentrated hydrochloric acid for 2 h. After placement at 4 °C for 24 h, the yellow liquid obtained was stirred with 300 mL of 50% pre-cooled ethanol for 2 h. Subsequently, 2% opaque colloidal chitin was obtained through centrifugation at 4000 rpm for 20 min. Afterward, deionized water was added until pH 6.0 was reached. For preparation of 3,5-dinitrosalicylic acid (DNS) aqueous solution, 6.3 g of DNS was dissolved in 262 mL of 2 mol/L NaOH solution. Subsequently, 182 g of sodium potassium tartrate,

5 g of steamed phenol, and 5 g of sodium sulfite were added to 500 mL of hot deionized water. The volume was adjusted to 1 L using pre-cooled deionized water to obtain DNS aqueous solution. For chitinase enzyme activity measurement, 200 μ L of 2% colloidal chitin, 300 μ L of Tris-Cl (pH 7.4), and 500 μ L of chitinase were added into a 1.5 mL of tube and reacted for 1 h at 37 °C. After cooling, the reacted liquid was centrifuged at 6000 rpm for 10 min. 1 mL of DNS was added to the supernatant, and the mixture was heated for 10 min in a boiling water bath. After the solution had cooled, the absorbance value was determined at 535 nm using a spectrophotometer (HITACHI 180-80, Japan). In parallel, the same volume of deionized water, instead of chitinase solution, was used as the negative control. One unit of chitinase activity was defined as the amount of enzyme that produces 1 μ mol of reducing sugar per 1 h at 37 °C. The chitinase concentration was determined using Coomassie brilliant blue method [31].

2.4. Preparation of surface SNPs and SNPCs

The preparation processes (Fig. 1) for surface-grafted SNPs and SNPCs generally followed previously described methods [32–34]. Briefly, for the surface-grafted SNP preparation, 500 mg of SNP1 [Fig. 1(a)] with hydroxyl groups was dispersed and suspended in 75 mL of deionized water. 1.5 mL of ammonia and 125 μ L of APTES were added to the SNP1 suspension and stirred for 2 h. The suspension was then centrifuged thrice at 14 000 rpm for 30 min. SNP2 [Fig. 1(b)] with amino groups grafted on the surface was obtained by drying the supernatant suspension. 500 mg of SNP2 was ultrasonically dispersed and suspended in a mixture of 30 mL of tetrahydrofuran, 0.1 g of succinic anhydride, and 100 μ L of triethylamine. This suspension was shaken for 12 h. Furthermore, SNP3 [Fig. 1(c)] with a carboxylic group-grafted surface was obtained through centrifugation and drying as described

above. Subsequently, SNP2 (500 mg) was dispersed and suspended in 20 mL of distilled water and 400 μ L of 25% glutaraldehyde. This suspension was shaken for several minutes until a pink color becomes apparent. SNP4 [Fig. 1(d)] with an aldehydic group-grafted surface was then obtained by centrifugation and drying. SNPC preparation using electrostatic adsorption involved the following: 25 mg of SNP1, SNP2, or SNP3 was dispersed and suspended in 4 mL of deionized water and 0.5 mg of Chi9602 for 12 h at 4 °C. Subsequently, SNPC1 precipitates [Fig. 1(e)] with Chi9602 adsorbed on the SNP1 surface [Fig. 1(f)], SNPC2 with Chi9602 adsorbed on the SNP2 surface, and SNPC3 [Fig. 1(g)] with Chi9602 adsorbed on the SNP3 surface were collected by centrifugation at 14 000 rpm for 20 min. SNPC preparation using covalent linkage: 25 mg of SNP3 was dispersed and suspended in 3 mL of deionized water; in addition, EDC and NHS were then added to the suspension. The final EDC concentration was 4 mmol·L⁻¹ and that of NHS was 1 mmol·L⁻¹). After reaction for 30 min at room temperature, the activated SNP3 precipitate with carboxyl groups was collected through centrifugation at 14 000 rpm for 20 min. The precipitate was dispersed and suspended in deionized water again, and 0.5 mg of Chi9602 was added and allowed to react for 30 min at 4 °C. The SNPC4 precipitate [Fig. 1(h)] with Chi9602 immobilized on the SNP3 surface was collected through centrifugation at 14 000 rpm for 20 min. 25 mg of SNP2 was dispersed and suspended in 4 mL of deionized water, and 0.5 mg of chitinase was added and allowed to react for 6 h at 4 °C. The SNPC5 precipitate [Fig. 1(i)] with Chi9602 immobilized on the SNP2 surface was collected through centrifugation at 14 000 rpm for 20 min. The chitinase immobilization rate was calculated using the following equation:

$$\text{Immobilization rate} = \frac{C_1 \cdot V_1}{C_0 \cdot V_0} \times 100\% \quad (1)$$

188

189 where C_0 and V_0 denote the initial concentration ($\mu\text{g/mL}$) and volume (mL) of
 190 Chi9602 prior to immobilization, respectively. C_1 and V_1 denote the Chi9602
 191 concentration ($\mu\text{g/mL}$) and volume (mL) after immobilization, respectively.

192

$$\text{Residual enzyme activity} = \frac{E_1 \cdot C_1 \cdot V_1}{E_0 \cdot C_0 \cdot V_0 - E_2 \cdot C_2 \cdot V_2} \times 100\% \quad (2)$$

194

195 where C_0 , V_0 , and E_0 denote the initial concentration ($\mu\text{g/mL}$), volume (mL), and
 196 specific enzyme activity (U/mg) of Chi9602 prior to immobilization, respectively. C_1 ,
 197 V_1 , and E_1 denote the concentration (mg/mL), volume (mL), and specific enzyme
 198 activity (U/mg) of SNPCs, respectively. C_2 , V_2 , and E_2 denote the concentration
 199 (mg/mL), volume (mL), and specific enzyme activity (U/mg) of the biocomposite
 200 SNPC2 supernatant after centrifugation.

201 **2.5. Characterization of the surface-modified SNPs and nanoscale chitinases**

202 The surface-grafted SNPs, SNP1, SNPC4, and SNPC5 were dried by vacuum
 203 freeze-drying. The products were mixed with KBr and pressed into tablets. FTIR
 204 spectrometry (Spectrum One; PerkinElmer, USA) was employed by scanning within
 205 the 400 cm^{-1} to 4000 cm^{-1} spectral range. SNP1, SNP2, and SNPC2 were also dried
 206 by vacuum freeze-drying. Their morphology was observed using a JSM-6390/LV
 207 scanning electron microscope (NTC, Japan) at 20 kV. Zeta potentials and particle
 208 sizes of all SNPs and SNPCs were determined using a Zetasizer Nano-ZS (Malvern
 209 Instruments, UK).

2.6. Bioassay of nematocidal activity

An overnight culture of *B. thuringiensis* YBT-020 was inoculated into 50 mL of fresh PM medium [35] and cultured at 28 °C and 400 rpm until approximately 70% spores and crystals were released (approximately 30 h to 32 h). The preparatory mixture of *B. thuringiensis* spore-insecticidal crystal protein (ICP) was collected, and the ICP content was quantified by following standard procedures (National Standard of the People's Republic of China, GB/T 19567.2-2004). About 1.00 g of *B. thuringiensis* YBT-020 spore-ICP powder was fully dissolved in 10 mL of sterile 0.01 M Na₂CO₃–NaHCO₃ buffer (pH 9.6) and subsequently diluted with sterile double distilled H₂O (ddH₂O) to 25.0 mL using a 25 mL volumetric flask to prepare a 40.0 mg/mL spore-ICP stock solution. These processes were performed to determine the half-lethal concentration (LC₅₀) against *Caenorhabditis elegans* wild-type strain N2 (Bristol, UK). The stock solution was further diluted to 1.0, 2.0, 5.0, 10.0, 15.0, and 20.0 mg/mL solutions with sterile ddH₂O. A total of 100 µL of each of the diluted solutions (including the stock solution) was added to different wells of a 96-well cell culture plate. These wells were further loaded with 50 µL of 80 µg/mL Chi9602 or nanoscale Chi9602, 5 µL of *C. elegans* larval (L4) suspension (containing a total of 40 larvae, and *C. elegans* synchronization method was followed Porta-de-la-Riva [36]), 5 µL of 10 mM 5-fluoro-2'-deoxy-uridine (Tokyo Chemical Industry, Tokyo, Japan), and 40 µL of *E. coli* OP50 cell suspension at 0.6 OD₆₀₀ nm. In parallel, 100 µL of each of the above solutions was added to wells of another plate loaded with similar solutions; however, 50 µL Chi9602 or nanoscale Chi9602 was replaced with 50 µL sterile ddH₂O. All samples were prepared in triplicate. All worms were incubated at 25 °C for 3 d. The worms were counted under an inverted microscope (OLYMPUS IX73, Japan) [37]. To evaluate the half-lethal time (LT₅₀) against *C. elegans*, all the

other conditions were similar; however, the concentration of *B. thuringiensis* YBT-020 spore-ICP was 10.0 mg/mL, and the worms were observed after 8, 16, 24, 32, 40, 48, 56, 64, 72, 80, 88, 96, 104, 112, and 120 h. All testing data were analyzed using the IBM SPSS statistics 19 software.

2.7. Immunofluorescence microscopy

Purified chitinase Chi9602 was used to prepared a polyclonal antiserum in a mouse model through a commercial service (Wuhan Boster Biological Engineering Co., Ltd., Wuhan, China). 200 μ L of 50 μ g/mL SNPC2, 770 μ L of 1% (w/v) BSA-PBS, and 30 μ L of Chi9602 antiserum (1/10000 diluted) were added into a 1.5 mL lucifugal centrifuge tube. After 90 min at room temperature, the precipitate of SNPC2, which contains Chi9602 antiserum, was collected through centrifugation at 14 000 rpm for 20 min), washed thrice with PBS, and subsequently dispersed and suspended in 1 mL of 1% (w/v) BSA-PBS. Cy3-IgG (second antibody, 2 μ L) (Wuhan Boster Biological Engineering Co., Ltd., Wuhan, China) was added into the SNPC2 suspension. After 90 min at room temperature, the precipitate of SNPC2, which contains Chi9602 antiserum and Cy3-IgG, was collected through centrifugation at 14 000 rpm for 20 min, washed thrice with PBS, and subsequently dispersed and suspended in 1 mL of 1% (w/v) BSA-PBS. This suspension (100 μ L) and 5 *C. elegans* N2 were added to a well of 96-well plate. After 12 h, the *C. elegans* N2 were gently pipetted out, cleaned thrice with deionized water, and immediately observed under a BX51 optical-fluorescent phase-contrast microscope (Olympus, Japan).

3. Results and discussion

3.1. Preparation and characterization of surface-grafted SNPs and SNPCs

Most NPs generally require surface modification and grafting with specific chemical groups before they can be used as nanocarriers [28]. As shown in Fig. 1, several SNPs, including SNP2, SNP3, and SNP4, were synthesized by grafting amino, carboxylic, and aldehydic groups, respectively, onto SNP1. SNP1 is rich and has different bonding states of silicon hydroxyl on its surface. For the preparation of biocomposite nanochitinases, purified Chi9602 was further loaded onto certain surface-modified SNPs. As a result, three SNPCs, i.e., SNPC1, SNPC2, and SNPC3, were obtained via Chi9602 electrostatic adsorption onto the SNP1, SNP2, and SNP3 surface, respectively. Meanwhile, SNPC4 and SNPC5 were obtained via Chi9602 covalent linkage onto the SNP3 and SNP4 surface, respectively.

FTIR spectroscopic analysis (Fig. 2) of the constructed SNPs and SNPCs was performed to verify the distinctive surface-bound groups. As shown in Fig. 2Aa, the peak at 3434 cm^{-1} in the SNP1 spectrum corresponded to the stretching vibration peak of Si-OH; the peak at 1101 cm^{-1} corresponded to the transverse and longitudinal symmetric contraction vibration peak of Si-O-Si; the peak at 806 cm^{-1} corresponded to the symmetric contraction vibration peak of Si-O-Si; and the peak at 470 cm^{-1} corresponded to the bending vibration peak of Si-O-Si. For the SNP2 spectrum (Fig. 2Ab), the new wave at 665 cm^{-1} denotes the stretching vibration peak of -N-H. In the SNPC4 spectrum (Fig. 2Ac), the new wave at 1158 cm^{-1} denotes the bending vibration peak of -C-C-. For the SNPC4 spectrum (Fig. 2Ad), no new peaks appeared. As shown in Fig. 2B, for the SNP4 spectrum (Fig. 2Be), the peak at 2925 cm^{-1} corresponded to the stretching vibration peak of -CH₂-. For the SNPC5 spectrum (Fig. 2Bf), the new wave at 2853 cm^{-1} corresponded to the stretching vibration peak of -CH₃, and that at 1466 cm^{-1} belongs to the bending vibration peak

of $-\text{CH}_3$. Therefore, the FTIR results confirmed in principle the successful SNP2, SNP3, and SNP4 grafting through amination, carboxylation, and hydroformylation.

Surface grafting was further confirmed by the altered zeta potentials of these SNPs and SNPCs because modification with surface groups can change the SNP shearing surface potential [33]. Figure 3 shows the zeta-potential distributions and values of the SNPs and SNPCs. At pH 6.25, considerable changes in surface potential were observed in all the measured samples. The SNP1 surface hydroxyl groups were probably ionized weakly, which led to a negatively charged surface (-18.2 mV). The SNP2 surface amino groups can accept H^+ in aqueous solution, which led to a positively charged surface (11.5 mV). The SNP3 surface carboxyl groups were ionized strongly, which led to a more negatively charged surface (-42.2 mV) compared with SNP1. The SNP4 surface aldehyde groups cannot be ionized or bound with any hydrogen proton, and the residual $-\text{OH}$ was ionized weakly. These outcomes led to a less negatively charged surface (-10.6 mV) in SNP4 compared with SNP1. Electrostatic adsorption and covalent linkage are very common methods of enzyme immobilization. After Chi9602 electrostatic adsorption, the SNPC1, SNPC2, and SNPC3 surface zeta potential was -33.8 , 25.7 , and -34.4 mV, respectively. After covalent linkage with Chi9602, the SNPC4 and SNPC5 surface zeta potential was -38.2 and 14.3 mV, respectively. The change in zeta potential further revealed that SNP surface modification and Chi9602 immobilization onto the SNPs were successful [32–34].

3.2. Dispersion and storage stability of SNPCs in the aqueous solution

The dispersion capacity of the as-prepared SNPs and SNPCs was evaluated through visual observation of the transparency and precipitation effects of the SNP/SNPC dissolved suspensions (Fig. 4). All the prepared SNPs exhibited good

dispersion and high transparency in 24 h ddH₂O-dissolved suspensions (Fig. 4A). After immobilization with Chi9602, the dispersion differed remarkably, as SNPC1 [Fig. 4, B(e)] and SNPC5 [Fig. 4, B(f)] retained high dispersion in the suspension, however the suspensions with the other three SNPCs were turbid and sedimentary [Fig. 4, B(g), (h), and (i)]. Furthermore, all the prepared SNPCs were stored for 7 d under room temperature conditions to evaluate the storage dispersion. The results showed that only SNPC2 [Fig. 4, C(k)] exhibited well-dispersed storage stability over the other tested SNPCs, which is an essential quality for drug delivery and agricultural applications [1, 11].

3.3. Characterization of the SNPC immobilization capacity and enzyme activity

B. thuringiensis Chi9602 belongs to a family of 18 glycosyl hydrolases that are capable of hydrolyzing the β -1,4-glycosidic bond of chitin to form *N*-acetyl-D-glucosamine. The conserved domain of Chi9602 has a modular structure, which is composed of an N-terminal chitin-catalytic domain (CCD), a fibronectin type-III-like domain (Fn3D), and a C-terminal chitin-binding domain (CBD) [29]. The specific activity of this enzymatic hydrolysis is an important indicator that chitinase has been used as an agricultural biocontrol agent [16, 17]. A range of *N*-acetyl-D-glucosamine concentrations was employed to determine the standard curve for the hydrolytic product of colloidal chitin (fitting linear equation: $Y=0.8543X+0.0140$, $R^2=0.9962$) (Fig. S2). As shown in Fig. 5, the various SNPCs exhibited significantly different chitinase immobilization rates, which ranged from 29.5% to 92.0%. In addition, the residual enzyme activity ranged from 3.0% to 94.1%. Interestingly, the SNPC4 and SNPC5 that are covalently linked through surface-carboxylation and surface-hydroformylation showed 87.7% and 92.0% chitinase immobilization rates, respectively, however their corresponding residual

enzyme activities were only 12.8% and 3%, respectively. This result indicated that although the SNPCs were obtained by covalent linkage have high chitinase immobilization rates on their surface, low residual enzyme activity is also observed. Such outcome could cause poor applicability of the SNPCs. This phenomenon is probably caused by the covalent linkage involved in the formation of new chemical covalent bonds, which may lead to Chi9602 degeneration and inactivation. In addition, the chitinase binding domain in the N-terminus of Chi9602 pulls on the chitin chain and contributes a continuous hydrolytic capacity [18, 19]. However, this domain was enclosed, thus limiting the specific enzyme activity (Figs. 1h and 1i). By contrast, although the SNPC1, SNPC2, and SNPC3, prepared by electrostatic adsorption, exhibited lower enzyme immobilization rates (55.84%, 80.24%, 29.46%, respectively), these SNPCs retained higher enzyme activities of 44.82%, 94.08%, 93.81%, respectively. These findings may have been caused by the mild conditions employed, which cannot cause enzyme degeneration. In addition, more chitinase binding domains were functionally exposed. Among these SNPCs, SNPC2 exhibited an overall superior capacity in terms of both enzyme immobilization rate and residual enzyme activity. Therefore, SNPC2 was chosen to test its morphology, enzymatic stability, and nematicide delivery capacity.

3.4. Morphological analysis of SNP1, SNP2, and SNPC2

Figure 6 shows the SEM images, particle sizes, and particle size distributions of SNP1, SNP2, and SNPC2. The dried SNPs (SNP1, SNP2, and SNPC2) are clearly visualized as spherical nanostructured materials, with an average particle size of approximately 40 nm to 50 nm (Fig. 6A). Meanwhile, in aqueous solution, all of these samples had an average particle size of approximately 80 nm to 100 nm, with a narrow particle size distribution (Fig. 6B). These findings suggested that SNP1, SNP2,

and SNPC2 had good dispersion in the aqueous solution after preparation. The phenomenon of the larger average particle size in aqueous solution is probably caused by the non-dispersibility of NPs and swelling from water absorption.

3.5. Effects of pH, temperature, and ultraviolet radiation on SNPC2 enzyme activity

Effects of pH, temperature, and ultraviolet radiation (UV) on the Chi9602 enzyme activity of SNPC2 and of free Chi9602 were comparatively assayed to determine if the nanoscale preparations altered the pH, temperature, or UV stability. Figure 7A shows that SNPC2 retained relatively higher activity at pH 5 to 8, with the highest activity at pH 7, which was consistent with free Chi9602. Interestingly, Fig. 7B shows that although SNPC2 showed lower enzyme activity within the temperature range of 25 °C to 45 °C, however it exhibited higher resistance to higher temperatures within the range of 55 °C to 75 °C, which probably resulted from the protective shield of SNP2 on the immobilized Chi9602. Moreover, Fig. 7C shows the specific chitinase activity of free Chi9602 and SNPC2 after exposure to UV (30 W) for 0 to 140 min at a 30 cm distance. The calculated half-life of the SNPC2 specific chitinase activity was 23.4 min, which strongly contrasted the obviously shorter half-life of free Chi9602 (by 15.1 min). In addition, this finding indicated that the Chi9602 UV resistance is improved after being immobilized onto SNP2.

Other SNPC2 properties include its average nanoscale particle size (98.7 nm), unaltered pH tolerance, enhanced resistance to higher temperature and UV irradiation, and improved dispersion and storage capacities compared with free Chi9602. Given the abovementioned properties, this biocomposite should be suitable for utilization as a nano-biopesticide delivery system.

3.6. Improvement of the synergistic nematicidal effect of Chi9602 on B.

***thuringiensis* YBT-020 preparations by SNP2**

B. thuringiensis strains have been characterized by their ability to kill not only certain insect pests, such as those from Lepidoptera, Orthoptera, Diptera, and Coleoptera, but also Nematoda worms [15, 18, 34]. SNPC2 was mixed with *B. thuringiensis* YBT-020 preparations and was subjected to bioassays that used *C. elegans* as the target organism to investigate if nanoscale chitinase endows improved nematicidal activity on *B. thuringiensis* preparations. As shown in Table 1, the *B. thuringiensis* YBT-020 preparations exhibited substantial nematicidal activity against *C. elegans*, with an LC_{50} of 12.87 mg/mL and an LT_{50} of 57.99 h. Meanwhile, free Chi9602 had no nematicidal activity against *C. elegans*. Interestingly, free Chi9602 exhibited a synergistic nematicidal effect with *B. thuringiensis* YBT-020 preparation, in which the LC_{50} of the mixture with YBT-020 preparation, in combination with Chi9602, was reduced to 8.63 mg/mL. Moreover, the LT_{50} was shortened by 6.80 h compared with the YBT-020 preparation alone. These phenomena may be due to the chitin hydrolysis by Chi9602, which resulted in the easier access of the *B. thuringiensis* YBT-020 toxin protein into the worms. In normalized bioassays, SNP2 did not show any synergistic nematicidal effect with *B. thuringiensis* YBT-020 preparation. However, SNPC2 exhibited a remarkably synergistic effect with *B. thuringiensis* YBT-020 preparation, in which the LC_{50} of the mixture with YBT-020 preparation, in combination with SNPC2, was reduced to 8.35 mg/mL. Furthermore, the LT_{50} was shortened by 12.04 h compared with YBT-020 preparation alone. Therefore, SNPC2 can improve the synergistic nematicidal effect of Chi9602 on *B. thuringiensis* YBT-020 preparation, especially shortening the lethal time, which is an important indicator of biopesticide persistence in the field [1].

3.7. Delivery capacity of nanoscale chitinase in *C. elegans*

Figure 8 shows phase-contrast micrograph images and fluorescence micrograph images of *C. elegans* N2 L4 larvae that were fed with Chi9602 and SNPC2 for 12 h. *C. elegans* was treated with the antigen–antibody complex of Chi9602, Chi9602 antiserum, and Cy3-IgG, which can be activated by 550 nm visible light and generate 570 nm red fluorescence. Figure 8b shows that no fluorescence was observed, which indicates the absence of antibody. Figure 8d shows minor red fluorescence distributed in the pharynx and uterus, which were probably enriched in chitin in the *C. elegans* body. Figure 8f shows that the antigen–antibody complex, which comprises SNPC2, Chi9602 antiserum, and Cy3-IgG, was extensively distributed in the *C. elegans* body.

C. elegans has been widely used as a model organism in the fields of genetics, developmental biology, neurobiology, and environmental biology. Moreover, multicellular *C. elegans* can also be used as a screening platform for nanodrug delivery systems in biomedicine [38, 39]. In the present study, the immunofluorescence assay results showed that Chi9602 can be carried by SNP2 and can be delivered to the whole *C. elegans* body. These findings indicate that SNP2 can probably alter cell membrane transport activity by increasing membrane permeability and/or eliminating special bio-barriers, which then allows the antigen–antibody complex of SNPC2, Chi9602 antiserum, and Cy3-IgG to diffuse and ultimately be carried to the whole *C. elegans* body [4]. Chi9602 is a proteic biocontrol agent with a 74.5 kDa Mw and a 5.88 *pI*. In addition, SNP2 possesses multiple positive charges on its surface. Therefore, the developed SNPC2 could also serve as a functional nanocarrier to deliver other macromolecular proteic biopesticides, particularly those with a *pI* less than 7.

4. Conclusion

The surface groups of hydroxylated nano SiO₂ were chemically modified through amination, carboxylation, and hydroformylation. These processes were followed by *B. thuringiensis* chitinase Chi9602 immobilization via covalent or statically adsorbed linkages to prepare several nanoscale chitinases. One of these nanochitinases, SNPC2, with an amino group-grafted surface and surface-immobilized Chi9602 through electrostatic adsorption, displayed multiple admirable properties, such as the highest specific enzyme activity, a relatively high immobilization rate, nanoscale average particle size (98.7 nm), good dispersibility and storage stability, wide pH tolerance, increased thermostability, and UV resistance. Moreover, SNPC2 exhibited an excellent capacity to deliver nematicidal preparations to control *C. elegans* in laboratory trials. Therefore, the developed nanoscale SNP2 could serve as a functional drug or biopesticide delivery system for extended agricultural or biotechnological applications.

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524 **Tables**

525

526 **Table 1.** Bioassays of mixed YBT-020 preparations against *C. elegans* L4 worms.

Sample	LC ₅₀ (95% confidence bounds ^{a)})	Linear regression equation (linearly dependent coefficient ^{b)})	LT ₅₀ (95% confidence bounds ^{c)})	Linear regression equation (linearly dependent coefficient ^{d)})
YBT-020	12.87 (11.16–14.88)	Y=0.194X–2.494 (0.959)	57.99 (54.83~61.15)	Y=0.033X–1.937 (0.953)
Chi9602	>1000	–	–	–
YBT-020+Chi9602	8.63 (7.78–9.55)	Y=0.219X–1.886 (0.971)	51.18 (47.94–54.36)	Y=0.033X–1.708 (0.954)
YBT-020+SNP2	12.93 (11.91~14.05)	Y=0.185X–2.388 (0.963)	55.30 (52.11–58.47)	Y=0.034X–1.856 (0.976)
YBT-020+ SNPC2	8.35 (7.51–9.26)	Y=0.226X–1.889 (0.968)	45.95 (42.64–49.15)	Y=0.033X–1.535 (0.954)

^a Concentration of *B. thuringiensis* YBT-020 preparation (mg/mL).

^b PROBIT model of lethal concentration.

^c Contact time of *B. thuringiensis* YBT-020 preparation against *C. elegans* (h).

^d PROBIT model of lethal time.

Figure captions

Fig. 1. Schematic illustration of SNP surface modifications and preparation of SNPCs.

(a) SNP1, (b) SNP2, (c) SNP3, (d) SNP4, (e) SNPC1, (f) SNPC2, (g) SNPC3, (h) SNPC4, and (i) SNPC5.

Fig. 2. FTIR spectra of SNPs and SNPCs: (A) SNPs and SNPC4; (B) SNPs and SNPC5.

Fig. 3. Determination of zeta potentials. (A) Zeta-potential distributions and values of SNPs. (B) Zeta-potential distributions and values of SNPCs. All samples are dispersed in deionized water (pH 6.25, T=25 °C).

Fig. 4. Dispersion and storage stability of SNPs and SNPCs. (A) Suspensions of SNPs after surface modification: (a) SNP1, (b) SNP2, (c) SNP4, and (d) SNP3. (B) Suspensions of as-prepared SNPCs: (e) SNPC1, (f) SNPC2, (g) SNPC3, (h) SNPC5, and (i) SNPC4. (C) Suspensions of SNPCs after storage for seven days: (j) SNPC1, (k) SNPC2, (l) SNPC3, (m) SNPC5, and (n) SNPC4.

Fig. 5. Measurement of specific chitinase enzymatic activity and chitinase immobilization rate of SNPCs.

Fig. 6. Morphological analysis of SNPs and SNPC2. (A) SEM images. (B) Particle size and distribution.

Fig. 7. Stability of SNPC2. (A) Effects of pH, (B) temperature, and (C) UV on the specific enzyme activity of SNPC2.

Fig. 8. Immunofluorescence investigation of delivery capacity through *C. elegans* N2 feeding. (a) and (b) are phase-contrast and fluorescence micrographs of *C. elegans* L4 larvae after feeding on Chi9602 for 12 h. (c) and (d) are phase-contrast and fluorescence micrographs of *C. elegans* L4 larvae after feeding on Chi9602 and antibody for 12 h. (e) and (f) are phase-contrast and fluorescence micrographs of *C. elegans* L4 larvae after feeding on SNPC2 and antibody for 12 h.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8