International Journal of Biological Macromolecules xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

# Preparation of nanoscale *Bacillus thuringiensis* chitinases using silica nanoparticles for nematicide delivery

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ARTICLE INFO

*Article history:*
Received 21 January 2015
Received in revised form 5 September 2015
Accepted 11 October 2015
Available online xxx

*Keywords:*
Silica nanoparticles
Surface grafting
Chitinase
Nanocarrier
Biocontrol agent

ABSTRACT

A series of amino, carboxylic, and aldehydic surface-grafted silica nanoparticles (SNPs) was prepared based on $SiO_2$ 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 $SiO_2$ 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 nematicidal effect of *B. thuringiensis* YBT-020 preparation against *C. elegans*, with a reduced $LC_{50}$ of 8.35 mg/mL and a shortened $LT_{50}$ 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.



## 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 biobarriers, 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 agrobiotechnological 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 agrobiotechnological 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 o f the nanoparticle carriers

*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.

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http://dx.doi.org/10.1016/j.ijbiomac.2015.10.030
0141-8130/

[14]. However, while SNPs have witnessed a remarkable increase in research on agricultural applications, the investigations of the feasibility of certain SNPs used for efficient delivery of the proteic biopesticides are limited. Therefore, studies that focus on cost-effective and practical nano-biopesticide delivery systems to control agricultural and forestry pests are still necessary.

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

NP preparation is involved in size reduction to nanoscale levels via top-down methods, such as milling, high-pressure homogenization, or sonication; whereas bottom-up processes involve reactive precipitation and solvent displacement [1]. In 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 $SiO_2$, $SiO_2$ 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 $SiO_2$ 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 Jill 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 of 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 4,000 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 μL of 2% colloidal chitin, 300 μL of Tris·HCl (pH 7.4), and 500 μL 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 6,000 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 μmol 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 μL 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 μL 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

Please cite this article in press as: X. Qin, et al., Int. J. Biol. Macromol. (2015), http://dx.doi.org/10.1016/j.ijbiomac.2015.10.030