Journal of Hazardous Materials 341 (2018) 248–256

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Comprehensive *in vitro and in vivo* risk assessments of chitosan microparticles using human epithelial cells and *Caenorhabditis elegans*

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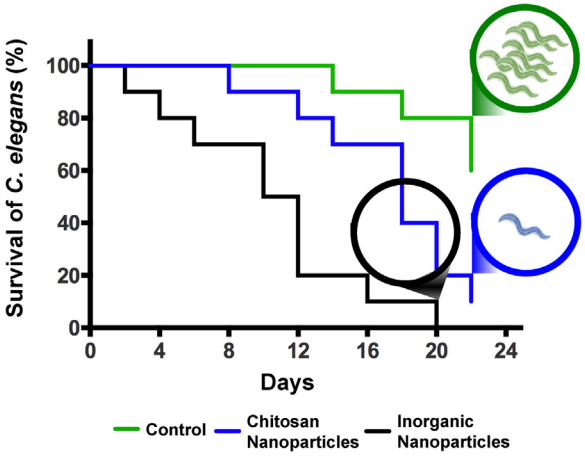
HIGHLIGHTS

- Chitosan microparticles do not cause cell toxicity at working concentration in human epithelial cells.
- However, chitosan microparticles show a subtle toxicity in *Caenorhabditis elegans* depending on types of CMs.
- *C. elegans* could be a sensitive animal model for risk assessments of nano- and microparticles.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 March 2017
Received in revised form 18 July 2017
Accepted 31 July 2017
Available online 1 August 2017

Keywords:
Chitosan microparticles
Risk analysis
Toxicity
Caenorhabditis elegans

ABSTRACT

The safety of using nano- and microparticles is a developing concern. In this study, we conducted risk assessments of chitosan microparticles (CMs) using *in vitro* human epithelial cell lines and *in vivo* animal model, *Caenorhabditis elegans*. After engineering of various CMs, we screened four CMs based on antimicrobial activity, which is a potential usage for disease treatment caused by multidrug resistant bacteria, and evaluated for risk assessments. CMs, with strong antimicrobial activity, and inorganic nanoparticles (SiO_2, TiO_2, and ZnO) did not cause toxicity in human cells measured by cell membrane integrity, mitochondria activity, and reactive oxygen species concentration. However, when applied to *C. elegans*, only CMs generated with low molecular weight chitosan and tripolyphosphate at 0.1% did not affect the lifespan, while the other CMs and inorganic nanoparticles shortened the lifespan, suggesting that they may cause subtle toxicity. These results suggest that *C. elegans* could be a sensitive animal model to measure low level of toxicity of nano- and microparticles. Taken together, although CMs do not cause toxicity at working concentrations of antimicrobial activity in human epithelial

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http://dx.doi.org/10.1016/j.jhazmat.2017.07.071

cells, they may cause toxicity at high concentration, suggesting that nano- and microparicles should be thoroughly investigated before they are applied.

1. Introduction

Chitosan, derived from chitin, is a linear polymer of β-(1,4)-linked N-acetylglucosamine [1]. It is Generally Recognized as Safe (GRAS) in many countries including Italy, Finland, Korea and Japan. Due to its non-toxic, biodegradable and biocompatible properties, it is applied in a broad area including biotechnology, in medicine and in agriculture. Chitosan facilitates to transport macromolecules across cellular barrier [2]. However, the action of native chitosan has been dependent on positively charged form at acidic pH in which chitosan poses activity [3,4].

Chitosan derivatives, such as chitosan microparticles (CMs) and nanoparticles, originally developed to enhance the functionality for drug carriers and gene transfer vectors [5], have been engineered by cross-linking of polysaccharide chitosan. Recently, it has been shown that CMs have antimicrobial activity against a broad spectrum of bacteria, including antimicrobial resistant microorganisms, without increasing the mutation rate of bacteria, providing significant insights into an alternative antimicrobial agent to treat infections caused by multi-drug resistant bacteria [6–8]. The mechanisms of antimicrobial activity of CMs are mediated by binding to bacterial surface molecules, including OmpA and lipopolysaccharide [8], and the antimicrobial activity has been proved to be active in different animal models [7,9,10]. Oral administration of CMs to cattle, which are a natural reservoir of the foodborne pathogen *Escherichia coli* O157:H7, showed significant reduction in the duration of pathogen colonization in the gastrointestinal tract without adverse side effect on animal health, evaluated by observing weight loss, decrease in feed consumption, diarrhea or abnormal behavior [9]. In addition, CMs have been applied to treat metritis in cows, which is caused by multiple infections of pathogenic bacteria in uteri. The *in vivo* antimicrobial activity of CMs was evaluated after direct infusion of CMs in the cow uteri, then the alteration of microflora in the uteri was measured using metagenomics analysis. It was found that CMs altered the sick uteri microflora to a more healthy composition, resulting in the increased treatment rate compared to the no treatment group [7].

The use of nanomaterials in various areas has been developing exponentially, but the toxicity risks associated with nanotechnology application have grown to become a major concern [6,11]. Although chitosan is widely regarded as safe because of its biodegradable and biocompatible features, the toxicity of CMs has not been investigated thoroughly. Although we have reported that CMs do not cause adverse side effects in large animal models [7,9], it is still possible that they are not sensitive enough to measure subtle effects that may occur during CM treatment, suggesting large animal models may not be ideal to evaluate CM's toxicity. Therefore, in this study, we evaluated the toxicity of CMs by measuring multiple cellular processes and in a small animal model.

Assays using cell lines are well accepted methodologies to assess the toxicity of different materials, including determination of cell membrane damage (lactate dehydrogenase [LDH] and Sytox Red staining assay), cell metabolic activity measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and cell apoptosis (Annexin V- Fluorescein isothiocyanate [FITC] staining assay) [12,13]. To evaluate the toxicity of nanomaterials, a combination of different assays is recommended to draw valid conclusions because nanoparticles may be redox active or adsorb dyes, which affect the results of spectrophotometer based assays [14]. *Caenorhabditis elegans* is commonly used to study toxicity because it has a high reproduction rate and can be easily maintained. They have transparent bodies and have complex tissues including intestinal, muscular, hypodermal, gonadal and nervous systems [15]. The transparency of the body makes it feasible to image *in vivo* and study the biodistribution and internalization of fluorescent nanoparticles [16], which are advantage to assess their toxicity [17]. In addition, the genome of *C. elegans* has been fully sequenced, allowing the study of biological processes at the molecular level. This model has used to assess toxicity of agents, including many inorganic nanoparticles [18–21]. For instance, it was reported that the LC50 of ZnO nanoparticles of *C. elegans* was 789 ± 103 mg/L and the EC50 s on movement and reproduction of *C. elegans* were 635 mg/L and 46 mg/L, respectively [19].

Here, we report the comprehensive evaluation of antimicrobial activity and toxicity of a different type of CMs, measured by disruption of multiple aspects of cellular processing including cell membrane damage, cellular metabolic activity and apoptosis as well as the CM toxicity in a small animal model, *C. elegans.* CMs showed no toxicity at working concentration to kill pathogens but moderate toxicity at higher concentration depending on the cellular processing assays, and *C. elegans* was a sensitive model for the evaluation of nanoparticle and microparticle toxicity.

2. Materials and methods

2.1. Preparation of chitosan microparticles

Chitosan microparticles (CMs) were prepared by an ionic gelation method as follows. A 2% (w/v) chitosan (low molecular weight chitosan [50–190 KDa and 75–85% deacetylated; LMWC] and high molecular weight chitosan [800 KDa and degree of deacetylation ≥ 75%; HMWC], Sigma-Aldrich, St. Louis, MO) solution was prepared with 2% acetic acid (v/v; Thermo Fisher Scientific Inc., Waltham, MA) and 1% Tween 80 (v/v; Acros Organics, Morris, NJ). For cross-linking, the chitosan solution was stirred and a cross-linker was added dropwise during 20 min of sonication (60 W). The sonication process was continued for another 25 min. Two types of cross-linkers, sodium sulfate (SS; Thermo Fisher Scientific Inc.) and sodium tripolyphosphate (TPP; Sigma-Aldrich), were used. The CMs were collected by centrifugation (8200*g*) after washing three times with sterile water. Four types of CMs were generated by using LMWC and HMWC with sodium sulfate (SS) and sodium tripolyphosphate (TPP).

2.2. Live/dead bacterial viability assay

To demonstrate the antimicrobial activity of CMs, a bacterial viability assay was conducted using the Live/Dead BacLight Bacterial Viability Kit (Molecular Probes, Inc., Eugene, OR). Briefly, 5×10^7 colony forming units per milliliter (CFU/ml) *E. coli* O157:H7 were inoculated into 1 ml of Mueller Hinton Broth containing 0.2% (w/v) of different CMs. The culture was incubated at 37 °C for 2 h and then incubated in the dark with SYTO 9 and propidium iodide for 15 min. The bacteria were observed using the fluorescence microscope (EVOS XL Cell Imaging System, Life Technologies, Carlsbad, CA).