



## Pharmaceutical Nanotechnology

## Chitosan nanoparticles and their Tween 80 modified counterparts disrupt the developmental profile of zebrafish embryos



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## ABSTRACT

Chitosan nanoparticles (CS-NPs) and their Tween 80 modified counterparts (TmCS-NPs) are among the most commonly used brain-targeted vehicles. However, their potential developmental toxicity is poorly understood. In this study, zebrafish embryos are introduced as an *in vivo* platform. Both NPs showed a dose-dependent increase in developmental toxicity (decreased hatching rate, increased mortality and incidences of malformation). Neurobehavioral changes included decreased spontaneous movement in TmCS-NP treated embryos and hyperactive effect in CS-NP treated larvae. Both NPs remarkably inhibited axonal development of primary and secondary motor neurons, and affected the muscle structure. Overall, this study demonstrated that CS-NPs and TmCS-NPs could affect embryonic development, disrupt neurobehavior of zebrafish larvae and affect muscle and neuron development, suggesting more attention on biodegradable chitosan nanoparticles.

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## 1. Introduction

With the increased application of nanoparticles in various fields such as biology (Wang and Wang, 2014), medicine (Zhang et al., 2008), biochemical engineering (Gupta and Gupta, 2005), and so on, it is expected that the possibility of the interaction between nanoparticles and human body is also going to increase. It was demonstrated in a review that nanoparticles could enter humans through several routes including ingestion, inhalation, dermal penetration, and injection, followed by the translocation of these nanoparticles towards secondary target organs and tissues via the lymph and blood circulation (Geiser and Kreyling, 2010; Stern and McNeil, 2008). Thus, the impact of nanoparticles on human health raises more and more concern.

Blood-brain barrier (BBB) is a tight and highly selective permeability barrier to prevent the entry of many substances into the brain, protecting the brain from injuries and diseases. However, due to their unique physicochemical properties such as small size and large surface area, nanoparticles have the ability to bypass the BBB and then exhibit potential toxicity to the brain (Hu and Gao,

2010; Sharma and Sharma, 2012; Wu et al., 2011). To date, with the development of nanomedicine, various nanoparticles are used as brain target drug delivery carriers for the treatment of many neurological disorders (Gao et al., 2013; Kong et al., 2012; Loureiro et al., 2014), but the assessment of the potential neurotoxicity induced by these nanoparticles are very few. Generally speaking, damage tissues have the ability to regenerate, while the self-regeneration of neurons is limited. Thus, the damage of the central nervous system (CNS) is irreversible. Hence, there is an urgent need for further investigation of the nanoparticle-induced neurotoxicity.

Due to the good biocompatibility, biodegradability, and simple preparation, chitosan nanoparticles (CS-NPs) are more attractive for non-viral gene delivery, controlled drug delivery, vaccine delivery, tissue engineering, as well as brain targeting better (Nagpal et al., 2010). Recently, nanotoxicity research mainly focused on inorganic nanoparticles, such as gold nanoparticles (Yang et al., 2014), metal oxide nanoparticles (Puzyn et al., 2011), multiwalled carbon nanotubes (Nagai et al., 2011). However, the nanotoxicity of biodegradable nanoparticles such as chitosan nanoparticles is still poorly understood. Chitosan, as a biodegradable organic polymer, is generally considered safe. But when chitosan is prepared as nanoparticles, the physicochemical properties change and the nanoparticles may accumulate in a certain tissue through circulation, thereby facilitating the harmful

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interactions between the nanoparticles and the biological system. Due to large surface area and small size, nanoparticles are easily entering into cells, while positive charge or additional surface modification may further enhance the cellular uptake (Hu and Gao, 2010). Additionally, these physicochemical properties may affect the *in vivo* distribution of the NPs (Hickey et al., 2015), and even disrupt the integrity of BBB (Lockman et al., 2004). Over dose of nanoparticle endocytosis cause oxidative stress and produce reactive oxygen species (ROS), which disrupt normal cell function and lead to cell death (Nel et al., 2006). ROS can also activate the MAPK signal pathway and induce inflammation (Kleinman et al., 2008). CS-NPs are usually cationic charged, which possess high cellular affinity and increasing cellular uptake. Several studies addressing the toxicity of CS-NPs both *in vitro* and *in vivo* have been reported (Hu et al., 2011; Park et al., 2013; Qi et al., 2005; Xu et al., 2009). As drug carriers, CS-NPs showed inhibitory effect on cancers, although the toxicity of CS-NP itself has also attracted concern. It was shown that chitosan nanoparticles elicited dose-dependent inhibitory effects on the tumor growth and induced tumor necrosis via the inhibition of tumor angiogenesis (Xu et al., 2009). There were reports indicated that CS-NPs (65 nm) exhibited apparent cytotoxicity against human gastric carcinoma cell line MGC803 (Qi et al., 2005). Therefore, the toxicity of CS-NPs for normal tissue is of great importance. Furthermore, researchers have recently found that after exposure morulae-stage embryos to CS-NPs (100 nm) *in vitro*, blastocyst complications and down-regulation of the expression of trophectoderm-associated genes and pluripotent marker genes were observed (Park et al., 2013). These results suggested potential developmental toxicity might be caused by CS-NPs. What's more, our previous study demonstrated that exposure of zebrafish embryos to CS-NPs with a particle size of 200 nm induced obvious bent spine in larvae (Hu et al., 2011), indicating potential developmental neurotoxicity as curved spine is one kind of the possible signal for CNS damage. However, the developmental neurotoxicity of CS-NPs has seldom been explored. The developing brain is extremely sensitive to neurotoxicants. Therefore, assessment of the potential developmental neurotoxicity of chitosan nanoparticles is required. Usually, CS-NPs have been modified with Tween 80 for brain targeting to treat brain disorders (Nagpal et al., 2013b; Trapani et al., 2011b). Our previous study taking rat as the *in vivo* model, showed that Tween 80-modified chitosan nanoparticles (TmCS-NPs) could enter into the brain after systemic injection, causing apoptosis and necrosis of neurons, and slight inflammatory response (Yuan et al., 2015). So, the developmental neurotoxicity of both CS-NPs and TmCS-NPs was evaluated in this study.

Conventional evaluation of developmental neurotoxicity in mammalian models is laborious and time consuming. The zebrafish (*Danio rerio*) has become an increasingly attractive vertebrate model for assessing developmental neurotoxicity, in part due to its small size, fecundity, embryonic transparency and its genomic and physiological similarity to mammals (DeMicco et al., 2010; Dishaw et al., 2014; Nishimura et al., 2014). The embryos of zebrafish can develop rapidly into larvae and most organs (especially brain) are fully functional at 5 days post of fertilization, allowing the assessment of developmental neurotoxicity in a short period. Shared basic structures and functional capacities have been demonstrated between the zebrafish brain and the human brain (Trobepe and Sive, 2003). In addition, since the embryo is transparent, it is possible to visualize development of the individual cells, central and peripheral nervous system through dyes, fluorescent tracer and gene probes. Moreover, externally developing embryos eliminate maternal toxicity in the toxicity assessments that require administration of the toxicant to the mother. The sensory pathways of zebrafish, such as vision, olfaction, taste, tactile, balance, as well as hearing, share an

overall homology with humans, leading to the development of several behavioural paradigms to assess the function of zebrafish (Xue et al., 2013). In summary, the zebrafish model has proven to be an ideal model for understanding the developmental neurotoxicity information at whole animal level.

The present study sought to investigate the developmental neurotoxicity of CS-NPs and TmCS-NPs. Here, CS-NPs and TmCS-NPs were prepared based on ionic gelation method and the development profile and developmental neurotoxicity were assessed using a zebrafish embryo model. We analyzed the effects of both CS-NPs and TmCS-NPs with a series of concentrations on zebrafish development profile and determined the LC<sub>50</sub> of the two nanoparticles. Afterwards, neurobehavioral assessments and corresponding mechanism studies were performed at a relatively lower exposure concentration (1/2 LC<sub>50</sub>). We found that both CS-NPs and TmCS-NPs could induce apparent development toxicity and neurobehavioral activity defects. Furthermore, embryos exposed to CS-NPs and TmCS-NPs elicited an enhanced generation of reactive oxygen species (ROS), inhibiting the development of the muscle and motor neurons at early stages of zebrafish, which might be responsible for the developmental neurotoxicity observed. We hope this study could provide new insights into the developmental neurotoxicity evaluation of biodegradable nanoparticles which are widely used in various fields.

## 2. Materials and methods

### 2.1. Materials

Chitosan (deacetylation degree, 86%; Mw, 100 kDa) was purchased from Zhejiang Jinke Biochemistry (Zhejiang, China). Sodium tripolyphosphate (TPP) was obtained from Shijiazhuang Shinerly Chemicals (China). Tween 80 was purchased from Sinopharm Chemical Reagent Co., Ltd (China). Reactive Oxygen Species Assay kit (KGT010-1) and Annexin V-propidium iodide apoptosis detection kit (KGA107) were bought from KeyGEN BioTECH (Nanjing, China). Acridine orange (CA1140, Solarbio, Beijing, China) was purchased from Beijing Solarbio Science & Technology Co., Ltd (China). Znp-1 (mouse monoclonal antibody; IgG2a, k) and zn-5 (mouse monoclonal antibody; IgG1, k) were obtained from Zebrafish International Resource Center, University of Oregon. Alexa Fluor 488 (P0188, Immunol Fluorescence Staining Kit with Alexa Fluor 488-Labeled Goat Anti-Mouse IgG), Tween 20 (ST825), and Triton X-100 (ST795) were purchased from Beyotime (Haimen, China).

### 2.2. Zebrafish embryos and embryo rearing

Eggs of AB strain zebrafish were obtained from College of Animal Sciences, Zhejiang University (Hangzhou, China). The author claims that all experimental protocols and procedures with live animals in this study were in agree with the relevant laws and guidelines, and the institutional committees approved our experiments. The fertilized embryos were collected within the first hour and then transferred into a culture dish with zebrafish culture medium (130 mg NaHCO<sub>3</sub>, 11.5 mg KCl, 120 mg MgSO<sub>4</sub>, and 444 mg CaCl<sub>2</sub> in 2 L ultrapure water). The medium in the breeding dish was renewed once a day. A stereomicroscope (Jiangnan, China) was used to observe the development of the zebrafish embryos and larvae.

### 2.3. Preparation and characterization of CS-NPs and TmCS-NPs

CS-NPs and TmCS-NPs were prepared according to the ionic gelation of chitosan with TPP anions under acidic condition. (a) CS-NPs: 100 mL of a chitosan solution (0.2%, w/v, in acetic acid 2%, v/v)

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